Tolerance of yeast biofilm cells towards systemic antifungals

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Publication date:
2014

Document Version
Peer reviewed version

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PhD thesis

by

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April 2014

Preface

This thesis is written as a partial fulfillment of the requirements to obtain a PhD degree at the Technical University of Denmark. The work presented in this thesis was carried out from April 2011 to April 2014 at the Department of Systems Biology and the National Veterinary Institute under the supervision of Associate Professor Anders Folkesson and Associate Professor Birgitte Regenberg. This work was funded by the Danish Agency for Science Technology and Innovation.

Rasmus Kenneth Bojsen
Vanløse, April 2014

Acknowledgements

I want to express my gratitude to my supervisors Anders Folkesson and Birgitte Regenberg for their outstanding guidance throughout my PhD project. You have both been very inspiring and always contributed with valuable inputs and excellent discussions.

I am especially grateful to David Gresham who taught me about barcode sequencing during my visit at his lab at New York University. Also thanks to all the members of the Gresham lab for the beers and exciting ping pong matches.

I would like to thank the past and present members of the antibiotic resistance group at IMG and VET for good discussions; Anders Folkesson, Nicholas Jochumsen, Vinoth Wigneswaren, Yang Liu, Linda Jensen, Sabine Pedersen, Martin Vestergaard, Mette Lund, Julie Claesen, Gunilla Veslemøy and Anders Mellerup.

I have enjoyed collaborating with the Regenberg lab and I would like to send my gratitude to Kaj Andersen, Rasmus Torbensen, Camilla Larsen and Henrik Møller for great scientific discussions and for contributing with some of the experimental work presented in this thesis. I really had fun at the Christmas parties and all the dinners.

I would also like to thank Lars Jelsbak and Søren Molin for your good advices and friendly attitudes. A special thanks to Martin Weiss for help with biofilm growth chambers and expertise in confocal microscopy. Thanks to the remaining past and present IMG members for contributing to a great work environment during the first two years of my PhD. A special thanks to the administrative and technical staff; Mette Munk, Pernille Winther, Janina Brøker, Lou Svendsen, Søs Koefoed, Lone Hansen and last but not least Lisser St. Clair-Norton who made dozens of YPD agar plates for me. Also a great thanks to Tomas Strucko for helping me with the GFP-tagging.

A special thanks to Mette Boye and the rest of the 3rd floor at VET for hospitality and kindness during the last year of my PhD when I had to move department with Anders. I have really enjoyed all the breakfasts and cake that we have consumed.

Last but not least I would like to thank my family and friends for support during the three years of ups and downs. Very special thanks to Trine soon-to-be-Bojsen for your love, patience and understanding.

Abstract

Fungal infections have become a major problem in the hospital sector in the past decades due to the increased number of immune compromised patients susceptible to mycosis. Most human infections are believed to be associated with biofilm forming cells that are up to 1000-fold more tolerant to antimicrobial agents compared to their planktonic counterparts. Antifungal treatment of biofilms will therefore often result in treatment failure. Consequently, there is a basic requirement to understand the underlying tolerance mechanisms and to development of novel anti-biofilm treatment strategies.

The focus of this thesis has been to explore the tolerance mechanisms of yeast biofilms to systemic antifungal agents and to identify the molecular target of a novel peptidomimetic with anti-biofilm activity. The genetic tractable *S. cerevisiae* was used as biofilm model system for the pathogenic *Candida* species in an attempt to take advantage of the molecular tools available for *S. cerevisiae*.

Mature biofilms containing mainly growth arrested cells were shown to be tolerant to three out of four tested antifungals, while all drugs had inhibitory activity against proliferating biofilm cells, demonstrating that drug treatment efficacy of biofilm cells is highly dependent on cellular growth phase. Similar results were obtained for planktonic cells, showing that ceased proliferation is a shared tolerance mechanism between biofilm and planktonic cells.

It was found that the membrane pore-forming agent amphotericin B was the only tested drug with activity against both growth arrested biofilm and planktonic cells but was found to only kill ~95 % of the cells. By using a collection of barcode tagged deletion mutants, we were identified that defects in protein synthesis, intracellular transport, cell cycle and lipid metabolism resulted in increased amphotericin B tolerance in both biofilm and planktonic cells. We furthermore observed that the tolerance level could be enhanced by nutrient starvation and inhibition of the TOR pathway.

In conclusion, antifungal tolerance is the combined effect of the physiological state of the cell and the mechanism of action of the drug, and this is independent of mode of growth. Based on these results, it can be suggested that future drug treatment strategies should focus on targeting growth arrested cells, rather than distinguishing between modes of growth.

At last, we analyzed the antifungal activity of the novel peptidomimetic LTX-109. We showed that this molecule rapidly killed yeast cells and that cell death was associated with release of protons, potassium and amino acids to the extracellular environment. Screening a yeast deletion collection for LTX-109 resistance indicated that complex sphingolipids were involved in fungicidal activity of LTX-109. The sphingolipids may therefore represent a unique antifungal target with therapeutic potential for future drug development.

Dansk resumé

Svampeinfektioner er blevet et stort problem i hospitalssektoren i løbet af de sidste årtier fordi andelen af patienter med nedsat immunsystem, der er følsomme overfor mycosis, er steget. Det skønnes at de fleste infektioner i mennesker er associerede med biofilmdannende celler, der er op til tusind gange mere tolerante overfor antimikrobielle stoffer sammenlignet med planktoniske celler. Der er derfor et behov for at forstå de underliggende tolerancemekanismer, samt udvikle nye anti-biofilm behandlingsstrategier.

Der har i denne afhandling været lagt fokus på at undersøge biofilmcellernes tolerancemekanismer overfor systemiske antifungaler, samt at identificere det molekylære target for et nyt peptidomimetic med anti-biofilm aktivitet. *S. cerevisiae* blev brugt som biofilm modelsystem for de patogene *Candida* arter i et forsøg på at udnytte de mange molekylære værktøjer, der er til rådighed for *S. cerevisiae*.

Modne biofilm, der hovedsageligt bestod af ikke voksende celler var tolerante overfor tre ud af de fire antifungaler som blev testet i dette studie, hvorimod alle stofferne havde inhibitorisk aktivitet overfor voksende biofilmceller. Disse resultater viste at aktiviteten af antifungalerne var afhængig af cellevækstfase. Lignende resultater blev observeret med planktoniske celler, hvilket viste at manglende vækst var en delt tolerancemekanisme mellem biofilm og planktoniske celler.

Det blev fundet at det membranporedannende stof amphotericin B var det eneste testet stof, der dræbte både ikke-voksende biofilm og planktoniske celler. Amphotericin B var dog kun i stand til at dræbe ~95 % af cellerne. Der blev derfor foretaget et barcode sekventeringsscreen af en blandet samling af gendeleterde mutanter for at bestemme de molekylære mekanismer, der resulterede i amphotericin B overlevelse. Det blev fundet at fejl i proteinsyntese, intracellulær transport, cellecyklus og lipid metabolisme førte til øget amphotericin B tolerance i både biofilm of planktoniske celler. Derudover observerede vi at toleranceniveauet kunne øges ved næringsstofsult on inhibering af TOR pathway.

Disse resultater viser at tolerance overfor antifungale stoffer er den samlede effekt af cellens fysiologiske tilstand og stoffernes virkemekanismer. Det kan på baggrund af disse data foreslås at fremtidige behandlingsstrategier skal fokusere på at ramme ikke-voksende celler i stedet for at skelne mellem biofilm og planktonisk vækst.

I den sidste del af afhandlingen blev det fundet at det nye peptidomimetic LTX-109 hurtigt dræbte gærceller, hvilket resulterede i udstrømning af protoner, kalium og aminosyrer. En samling af gendeleterede mutanter blev screenet for at identificere LTX-109 resistente mutanter, hvilket viste at komplekse sfingolipider var involverede i den fungicide aktivitet af LTX-109. Sfingolipiderne er unikke antifungale targets med stort potentiale for udvikling af fremtidige behandlingsstrategier.

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Publication list

Bojsen, R.*, Torbensen, R.*, Larsen, C. E., Folkesson, A., and Regenberg, B. (2013). The synthetic amphipathic peptidomimetic LTX109 is a potent fungicide that disturbs plasma membrane integrity in a sphingolipid dependent manner. *PLoS One, 8(7): e69483*

Bojsen, R., Regenberg, B., and Folkesson, A. (2014). Yeast biofilm tolerance towards systemic antifungals depends on growth phase. *Manuscript submitted to J Antimicrob Chemother*

Bojsen, R., Regenberg, B., Gresham, D., and Folkesson, A. (2014). Cellular state determines amphotericin B tolerance in yeast biofilm and planktonic populations. *Manuscript in preparation*

Not included in thesis

Bojsen, R. K., Andersen, K. S., and Regenberg, B. (2012). *Saccharomyces cerevisiae* - a model to uncover molecular mechanisms for yeast biofilm biology. *FEMS Immunol Med Microbiol, 65(2):* 169-82

Andersen, K. S., **Bojsen, R. K**., Sørensen, L. G., Nielsen, M. W., Lisby, M., Folkesson, A., and Regenberg, B. (2014). Genetic basis for *Saccharomyces cerevisiae* biofilm in liquid medium. *Manuscript submitted to G3 (Bethesda)*

Møller, H. D., **Bojsen, R.,** and Regenberg, B. (2014). Genome-wide purification of extrachromosomal circular DNA from eukaryotic cells. *Manuscript in preparation*

* Authors contributed equally

Abbreviations

AMPs	Antimicrobial peptides
Bar-seq	Barcode sequencing
DNA	Deoxyribonucleic acid
EGO	Exit from rapamycin-induced growth arrest
ESR	Environmental stress response
FDA	Food and drug administration
KanMX	Kanamycin resistance gene
MIC	Minimal inhibitory concentration
mRNA	Messenger RNA
PCR	Polymerase chain reaction
PKA	Protein Kinase A
QS	Quorum sensing
RNA	Ribonucleic acid
SAPK	Stress-activated protein kinase
SGA	Synthetic genetic array
TOR	Target of rapamycin
TORC1	TOR complex 1
TORC2	TOR complex 2

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1. Introduction

Microbial biofilms have been thoroughly studied since Bill Costerton and colleagues linked biofilm formation to human disease in the early 1980s and it was observed that biofilm cells can survive high concentrations of antimicrobial agents. Despite decades of research, the molecular mechanisms underlying drug tolerance of biofilm cells are not fully understood. Several mechanisms have been proposed to be involved, but none of them can solely account for treatment failure. This research subject is therefore highly relevant and extensively investigated. As of April 2014, PubMed listed >2400 publications in which the words “biofilm” AND “resistance” appear in “title/abstract” and the number of publications are increasing every year (Figure 1).

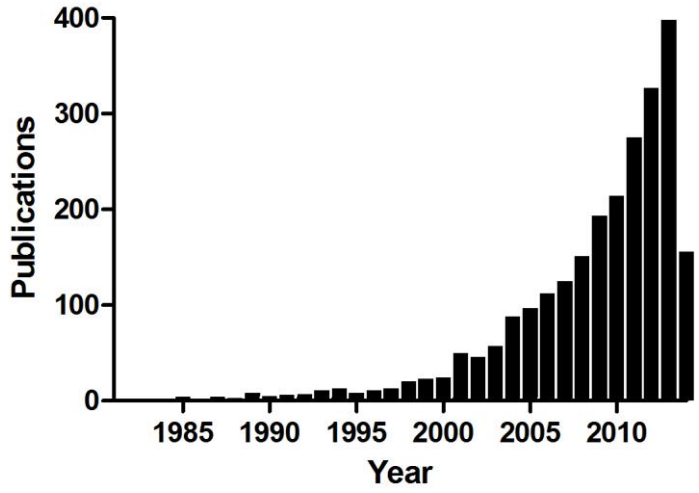

Figure 1. Number of publications on biofilm resistance. The number of publications containing the words "biofilm" and "resistance" in the title and/or abstract increase every year. Data from (pubmed.com).

The major subject of this thesis was to investigate the multidrug tolerance mechanisms of microbial biofilm cells. The research was performed in yeast that has become a major nosocomial problem during the past decades. Although biofilms caused by *Candida* species were among the first to be observed on invasive medical devices in the clinic [1], knowledge about yeast biofilms is limited compared to bacterial biofilms, possible due to the lack of genetic and molecular tools available for pathogenic fungi [2,3]. We therefore aimed at developing a model system using *Saccharomyces cerevisiae* to study yeast biofilms and take advantage of the molecular tools available for this fungus.

1.1 The term "biofilm"

Early publications of the word "biofilm" originate from marine microbiology that used the term to describe microbes trapped in wastewater filters in aquatic environments [4,5]. "Biofilm" was used in relation to human infection for the first time in 1984 [1], but Anthony van Leeuwenhoek has retrospectively been acknowledged as the first scientist to publish observations of biofilms with his microscopic investigations of aggregating cells on tooth surfaces (published in 1684) [6,7]. Research in the 1970s and 80s pioneered by Bill Costerton led to the classic definition of a biofilm: "*a structured community of* [...] *cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface*" [8]. Development and implementation of advanced microscopic techniques and transcriptomic analysis in biofilm research expanded the definition of biofilms to include that phenotypic profiles must be distinct from their planktonic counterparts [9]. Massive attention has been on antimicrobial recalcitrance of biofilm cells in the past decades. Although originally not a defining criterion for biofilms, the resistance to high concentrations of antimicrobial agents is today considered as one of the major hallmarks of microbial biofilms [10-12].

Recent *in vivo* research have led to an updated definition of biofilm that matches the lifestyle of infectious microbes observed in the clinic with aggregation and tolerance to antimicrobial agents as the two major biofilm characteristics [13]. With this definition it is no longer necessary to be surface attached or be encapsulated in a self-produced matrix as was dictated by the early biofilm dogma. Today, "biofilm" is used to describe several *in vitro* modes of growth including colonies on solid agar [14,15], free-floating aggregates [16] and attachment to an abiotic surface in liquid environments [17]. The latter *in vitro* model system has been used in this thesis for biofilm cultivation.

2. *Candida* infections

Candida is a genus of yeasts belonging to the kingdom fungi. *Candida* species can be isolated from soil and aquatic environments, but are also commonly found as a member of commensal human microbiota including the oral cavity, skin, gastrointestinal and genitourinary tract [18]. *Candida* species are opportunistic pathogens that can cause infections ranging from superficial to life-threatening systemic disease (Figure 2). Young and healthy individuals rarely become infected with fungi, while infants and the elderly are more at risk [19]. *Candida* are frequently isolated from immune compromised patients with HIV/AIDS, cancer, invasive medical devices or severe burn wounds [20]. Improvements in modern medicine and changes in medical practice have led to an extensive use of invasive medical devices, cancer chemotherapy and broad spectrum antimicrobials, which simultaneously have increased the population at risk of fungal infections [20,21].

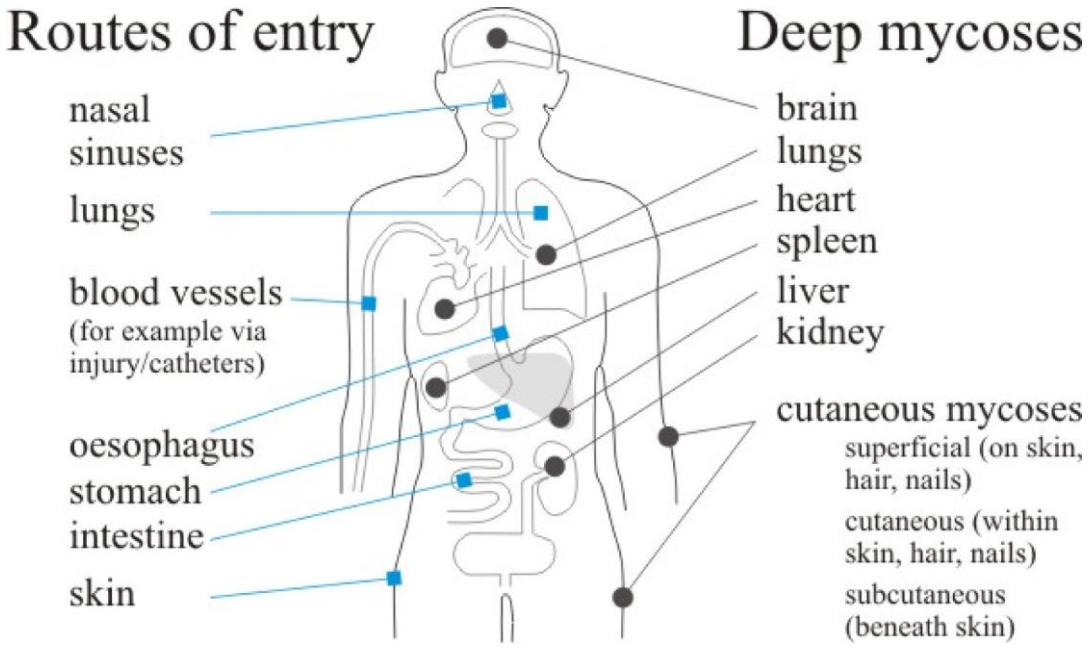

Figure 2. Routes of entry and distribution of fungal infections in humans. Pathogenic fungi can enter the human host at diverse sites causing infections ranging from superficial to deep seated mycosis. Figure adapted from Moore *et al.* (2011) [22].

Candida is the most frequently isolated fungal species from patients with fungemia [19] and it is now accepted as a major human pathogen in the nosocomial sector. *Candida* is the fourth leading cause of hospital acquired blood stream infections and catheter-related infections in USA with mortality rates at 40% [23,24]. Furthermore, *Candida* causes oral thrush in the majority of AIDS patients [25].

C. albicans is the most prevalent among fungal blood isolates accounting for approximately 50% of the incidences [26,27], but an increase in non-*Candida albicans* species have been reported in the past decades. This might be a result of the extensive use of fluconazole that has selected for intrinsic resistant *Candida* species such as *C. glabrata* and *C. krusei* [20,28,29].

2.1 Clinical significance of yeast biofilms

Dental plaques on tooth surfaces was one of the first examples of microbial biofilms to be recognized by the nosocomial sector [21], and it is presently estimated that most human infections are associated with biofilm formation [30-32]. Yeast infections are often associated with invasive medical devices that serve as a compatible surface for cell adhesion and biofilm formation. The increase in C*andida* infections has almost paralleled the increased use of medical implants in immune compromised patients [33,34]. Fungal biofilms can form on almost any medical device including catheters, prostheses, implants and cardiovascular devices [21,33]. The invasive device can be contaminated by the microflora of the patient or externally by i.e. hands of nursing staff [2,21]. The cells in a biofilm can cause chronic infection at the attachment site or they can detach from the biofilm and cause infection at other sites of the human body [35].

2.2 Biofilm development

Yeast biofilms develop in four stages that occur overlapping rather than sequential [36] (Figure 3). The initial step is attachment of single cells to a surface through non-specific interactions followed by the expression of specific adhesins for stronger attachment [20]. Yeast biofilm adhesins are cell surface glycoproteins rich in the amino acids serine and threonine [36]. Next, the yeast cells proliferate and colonize the surface in aggregates, while secreting an extracellular matrix. The matrix has been suggested to support surface adhesion, facilitate stress tolerance and create reservoirs for nutrients and waste products [37,38]. The extracellular matrix consists mainly of carbohydrates and proteins that accumulate in the maturation process and lead to a well-organized multicellular structure with extensive spatial heterogeneity. The mature biofilm structure depend on environmental conditions, while the amount of extracellular matrix material and cell morphology differentiate between the *Candida* species [20]. In the last step, cells disperse from the biofilm with the ability to infect various sites in the host as planktonic cells or form a new biofilm at another location [36].

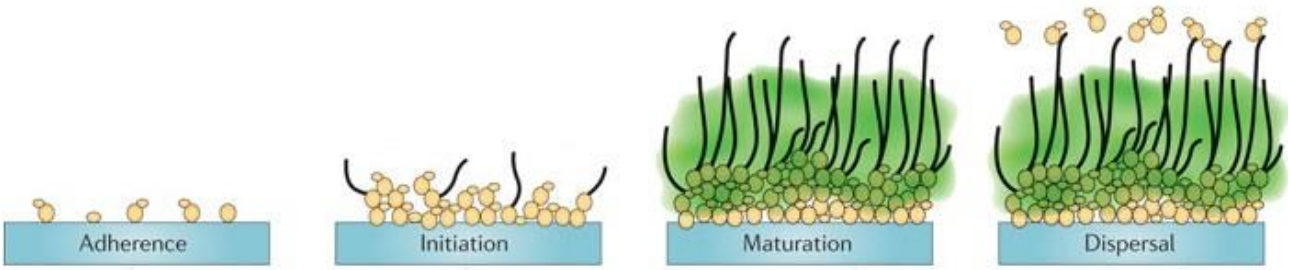

Figure 3. *Candida* biofilm development. In the first step of biofilm formation, planktonic yeast cells attach to a surface using adhesins. Next, the yeast cells proliferate and colonize the surface in aggregates forming a multicellular community. Some *Candida* species form hyphae while others only consist of yeast morphology. The biomass increases in the maturation process and the cells excrete an extracellular matrix that encapsulates the biofilm cells. The yeast cells can disperse from the biofilm and form a new biofilm at another site or cause infection as planktonic cells. Figure modified from Finkel and Mitchell (2011) [36].

Microbial cells can coordinate gene expression by sensing the accumulation of self-produced quorum sensing (QS) molecules in the environment. QS signaling is known to be a key regulator of bacterial biofilm formation [39], but less is known about their role in yeast biofilms. Several QS molecules have been observed to accumulate in mature yeast biofilms [36]. Furthermore, addition of the autoinducer farnesol results in disruption of the biofilm structure, suggesting a role of this molecule in biofilm stability and formation [40,41].

2.3 Treatment of biofilm infections

The link between fungal infections and biofilm formation originates from the detection of surface attached fungi on medical invasive devices after removal from the patient [42]. However, there is currently no diagnostic test available for clinicians to detect biofilm infections. Instead, several observations have been suggested to indicate biofilm associated infections: clinical symptoms of fungal infection, antifungal treatment failure or recurrent infections, the use of indwelling devices, and microscopic detection of multicellular aggregates [43].

There are four major drug treatment options for biofilm-associated fungal infections: antifungal mono and combination therapy, prophylaxis and surgical removal of biofilm-infected implants.

2.3.1 Antifungal monotherapy

Fungal infections are treated with systemic antifungals from four major drug classes (Figure 4).

Azoles

The azoles are a group of antifungal agents that target the cytochrome P450 enzyme lanosterol-14α-

demethylase. The demethylase is encoded by the gene *ERG11* and is responsible for the conversion of lanosterol to ergosterol. Inhibition of this enzymatic step results in ergosterol depletion and accumulation of toxic ergosterol procurers and the cell consequently cease proliferation [29,44]. The activity of most azoles is considered fungistatic. Azoles can be divided into imidazoles (miconazole, ketoconazole, clotrimazole), which are used in topical treatment and triazoles (fluconazole, itraconazole, voriconazole, posaconazole) that are developed for systemic treatment. The triazoles are the most frequently used drugs for treatment of mild to moderate systemic mycosis because of their favorable pharmacokinetic and pharmacodynamic properties [44,45].

Polyenes

The polyenes (amphotericin B and nystatin) are produced by *Streptomyces* and have been used in the clinic since the 1950s. They target ergosterol in the fungal cell membrane where binding alone is sufficient for antifungal activity because it inhibit vital processes controlled by ergosterol [46]. Another mechanism of action is pore-forming aggregation of polyenes in the lipid bilayer that facilitate efflux of ions and larger electrolytes [44,47]. Amphotericin B is the dominating polyene in clinical practice and it has fungicidal properties that result in rapid cell lysis. Amphotericin B has a broad spectrum of activity and is the gold standard for treatment of life threatening systemic infections [47,48]. The high homology of the fungal ergosterol to the human cholesterol results in severe toxic side effects from amphotericin B use. However, several amphotericin B formulation have been developed to limit the toxic side effects [49].

Flucytosine

The fluoropyrimidine 5-flucytosine is a pro-drug that is transported into the cell by cytosine permease. Conversion of the drug to fluorodine triphosphate can be incorporated into RNA in place of uridylic acid and result in inhibition of protein synthesis. Another mechanism of action is the processing of 5-flucytosine to 5-fluorodeoxyuridine monophosphate, which acts on thymidylate synthase to inhibit DNA synthesis [50,51]. The effect of flucytosine is growth inhibition of susceptible fungal cells [44]. Monotherapy with flucytosine is limited because of a high frequency of resistance development. Consequently, the use of flucytosine is often restricted to combination therapy with amphotericin B or triazoles [51].

Echinocandins

The echinocandins (caspofungin, anidulafungin, micafungin) consist of a family of lipopeptides that inhibit the cell wall β-1,3-D-glucan synthase complex. The echinocandins target the catalytic subunit Fks that is encoded by the genes *FKS1, FKS2* and *FKS3* leading to disruption of cell wall structure and osmotic

instability [44,52]. Treatment with echinocandins cause cell lysis and the drugs are fungicidal against *Candida* species and fungistatic against *Aspergillus* species [52,53]. The echinocandins have a narrow spectrum of activity and their use are often limited to invasive candidiasis [52].

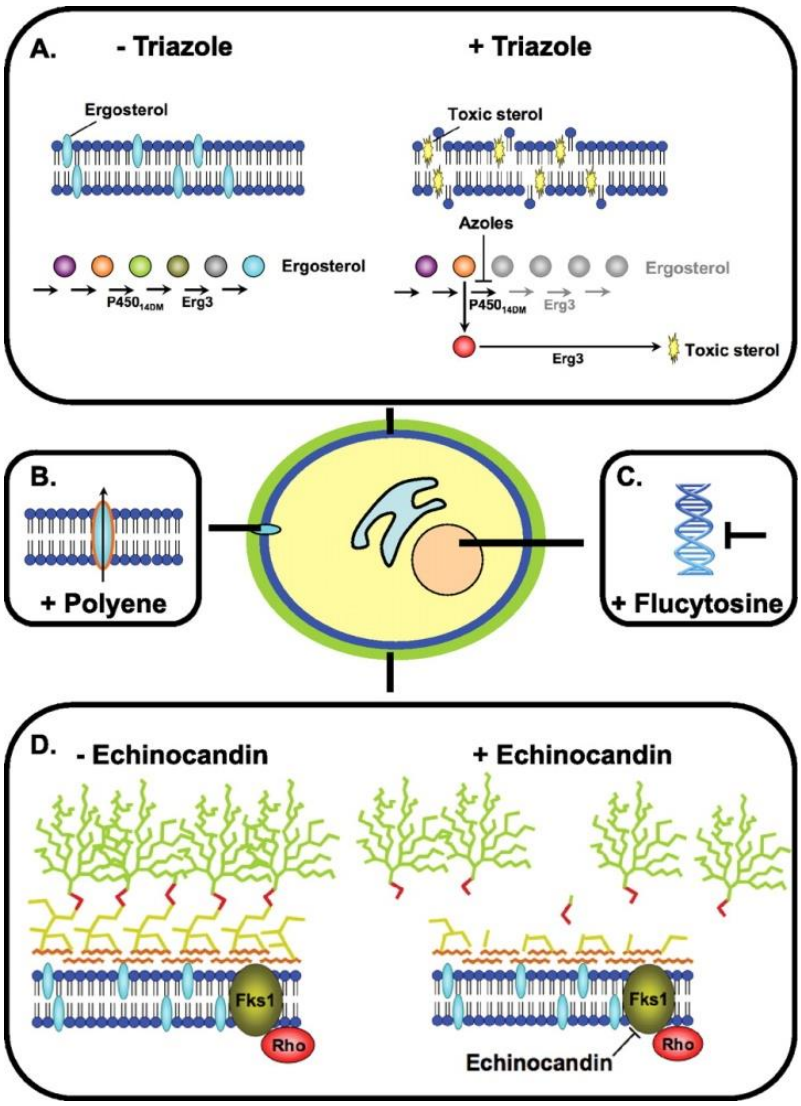

Figure 4. Mechanism of action of antifungal agents. (A) The triazoles inhibit ergosterol biosynthesis by binding to cytochrome P450, which cause accumulation of toxic sterols. (B) The polynes bind to ergosterol in the cytoplasmic membrane and form pores that lead to efflux of intracellular ions and electrolytes. (C) Flucytosine inhibits DNA and protein synthesis. (D) The echinocandins target Fks1 of the cell wall β-1,3-D-glucan synthase complex and inhibit cell wall synthesis. Figure adapted from Cowen and Steinbach (2008) [54].

2.3.2 Antifungal combination therapy

Combination therapy with two or more antifungal drugs can be used if they have synergistic effect. But it can also be used to decrease the concentration of amphotericin B to reduce the risk of toxic side effects, or to limit resistance development associated with flucytosine use [42,43]. One advantage of combination therapy can be to simultaneously target several biofilm subpopulations that each might express antifungal-specific resistance mechanisms.

2.3.3 Prophylaxis

Biofilm formation on medical invasive devices can be inhibited by prophylactic treatment of the device. This can be done by sterilizing the device by flushing it for hours with an inhibitory agent, or the device can be coated with an agent that prevents binding to the device. Antifungal lock therapy is another solution where a high concentration of an antimicrobial agent is introduced to the lumen of the catheter and “locked” for extensive time without being in use [10,42,43]. The goal is to reduce the risk of colonizing the catheter with a pathogen, but development of antimicrobial resistance is a major concern [10].

2.3.4 Removal of invasive device

The most efficient treatment of a device-related biofilm infection is to replace or remove the implant. This procedure is not always desirable, because of patient inconvenience and often life-supporting function of the medical implants [10,42,43].

3. Antifungal resistance

3.1. Inherited resistance

Failure to eradicate a fungal infection by antifungal treatment can be attributed to microbial or clinical resistance mechanisms. Microbial resistance refers to the inactivity of an antifungal drug against an unsusceptible fungus and can be intrinsic or acquired [55,56]. The fungus is intrinsic resistant to an antifungal agent if it can resist the drug without prior exposure. Intrinsic resistance can be observed in *C. glabrata* and *C. krusei* to some azoles and in *Cryptococcus neoformans* to echinocandins [56,57].

Acquired resistance is the development of resistance in a susceptible strain. Mutations in antifungal drug targets that result in decreased drug affinity are the most common resistance mechanism and can be applied to most antifungals. Mutations in *ERG11* that encode the target of azoles prevent binding to the enzymatic site. Similarly, mutations in *FKS1* inhibit the binding of echinocandins to the β-1,3-D-glucan synthase complex. Resistance to polyenes is often a result of mutations in the ergosterol biosynthetic pathway genes that result in the accumulation of ergosterol intermediates in the cell membrane [56]. *C. albicans* has two major efflux pump families that are encoded by the *CDR* and *MDR* genes and upregulation of these genes increase drug export out of the cell [56]. Only the azoles are substrate to *Candida* species efflux pumps, while no efflux pumps have been observed to transport the polyenes or echinocandins out of the cell [29,56,58]. Resistance to flucytosine is mediated by mutations in cytosine permease or cytosine deaminase that are responsible for uptake and conversion to the active compound, respectively [51,57]. Enzymes that interfere with antifungal activity have not been discovered in fungi [59] and intact fungi are unsusceptible to horizontal gene transfer without cell fusion [57]. Evolution of antimicrobial resistance in fungi is therefore a result of local mutations. Mutations that result in antifungal resistance are drug specific and observations of multidrug resistant strains in the clinic are extremely rare [29].

Clinical resistance is the persistence of infection despite antifungal treatment and includes survival of susceptible strains [55]. Such treatment failure can be caused by wrong diagnosis, the underlying disease of the patient, pharmacokinetics or pharmacodynamics, and the physiological state of the cell such as those in biofilms [56,57,60].

3.2 Tolerance of fungal biofilms to antifungal agents

The ability of microbial biofilms to survive treatment with high doses of antimicrobial agents is attributed to tolerance mechanisms rather than inherited resistance [61,62]. In contrast to the classic definition of antimicrobial resistance, which can be acquired by mutations of chromosomal genes or uptake of external DNA, non-inherited resistance is caused by phenotypic variations of susceptible cells [63]. Such non-inherited resistance also known as persistence will for consistency in this thesis be referred to as tolerance.

Antimicrobial drug tolerance has been given little attention compared to genetically altered resistance, although the importance of such non-inherited survival mechanisms is well documented and could develop into inherited resistance [61,63]. *Candida* biofilms have been suggested to possess several antifungal drug tolerance mechanisms that will be discussed below.

Extracellular matrix

One of the hallmarks of biofilm cells is the production of an extracellular matrix. One appealing suggestion is that it prevents the antimicrobial agents from reaching their targets either by physical blocking the penetration or by interactions between the antimicrobial drug and extracellular matrix components. Such shielding would depend on the physiochemical properties of the matrix and drug, and will not be applied for all drug classes because of the chemical diversity and molecule size of antimicrobial agents [64]. Research on *Candida* species biofilms with antifungal agents have shown that drug inactivity is not dependent on the amount of matrix produced by biofilm cells [65], and that the antifungal agents can penetrate the matrix layer in concentrations that kill exponential growing planktonic populations [66]. It has therefore been concluded that the matrix material does not play a significant role in antifungal tolerance of fungal biofilm cells [65,66]. The matrix layer may, however, have a much more significant role in surviving exposure to immune cells during infection [67].

Efflux pumps

Efflux pumps are common resistance mechanisms to the azole drug fluconazole in planktonic *Candida* populations. Since fluconazole has poor activity against *Candida* biofilms, this has led to the investigation of the involvement of major efflux pumps in biofilm associated drug tolerance. While upregulation of efflux pumps have been reported in the early and intermediate phases of biofilm development, but lost in the mature phase [68,69], others find them to be upregulated during all growth phases of biofilm development [70]. Despite this inconsistency, there is an agreement that efflux pump gene knockouts remain fluconazole tolerant [68,69,71] and efflux pumps can therefore not solely be responsible for the observed drug tolerance in biofilm cells. Furthermore, since the polyenes and echinocandins are not substrates to any known efflux pumps, they cannot account for the multidrug tolerance to these drugs [58].

Decreased drug target levels

Tolerance of biofilm cells might be achieved by decreased drug target levels. It has been observed that intermediate and mature phase biofilm cells have decreased membrane ergosterol levels [69], which result in fewer target molecules for the polyenes [72].

Slow growth

Biofilms typically inhabit nutrient limited environments and the starvation conditions result in decreased growth rate and metabolism of the biofilm cells. Especially cells located in the inner layer of biofilms have poor nutrient access and consequently have reduced cellular metabolism [73]. The inhibitory effect of antimicrobial agents is anti-correlated to growth rate and is highly dependent on cell division [63,74-76]. Accordingly, most systemic antifungals have activity against proliferating biofilm cells [77], but are inactive against mature biofilms [78].

Heterogeneity

Biofilm populations have a high level of phenotypic heterogeneity that can be attributed to nutrient and oxygen gradients across the biofilm [73,79]. Sub-populations may use different tolerance mechanisms and therefore, none of the mechanisms described above can individually account for the multidrug tolerance observed in biofilm cells. It is possible that a combined effect of each tolerance mechanism contribute to the overall tolerance, hence, drug treatment can only eradicate the entire biofilm population if it targets all sub-populations.

4. Stress tolerance

4.1 Phenotypic heterogeneity

Phenotypic variation between microbial cells in isogenic populations increases fitness in fluctuating environments. Heterogeneous populations are likely to contain cells that are more tolerant to acute stress exposure than homogeneous populations adapted to constant environments [80,81]. Several factors can contribute to phenotypic heterogeneity that results in diverse gene expression patterns among individual cells. Each cell cycle phase is associated with specific transcriptional activity and asynchronous cell cycle progression of individual cells will therefore result in gene expression variations [82]. The rate of cell cycle progression is correlated to cell age and replicative older cells with slow cell cycle progression will therefore have distinct gene expression compared to replicative younger cells [80,83] (Figure 5). Indeed, cell cycle phase, growth rate and cell age have all been linked to increased tolerance to environmental stress [80,82,83].

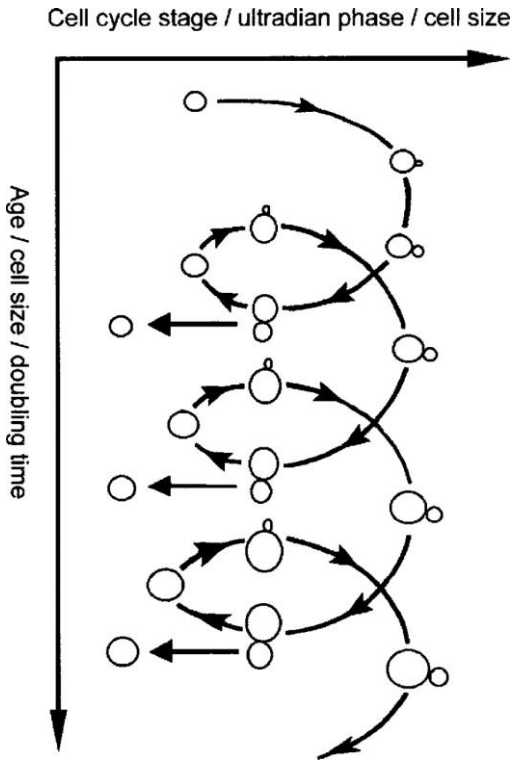

Figure 5. Population heterogeneity. Individual cells in a population divide asynchronously and result in cells with age and cell cycle stage variations. Cell cycle progression and cell age can drive population heterogeneity because different gene expressions are associated with cell cycle stage, growth rate and age of the cell. Figure adapted from Sumner and Avery (2002) [82].

Population heterogeneity can also be generated by stochastic variation in gene expression. Such stochasticity can be the result of protein or mRNA abundance diversity among individual cells that is caused by random synthesis and degradation rates. Gene expression can occur in pulsatile bursts, which give episodic protein synthesis in oppose to a constitutive gene expression. Translational bursting is a stochastic event that generates random variation in molecular processes during protein synthesis and cell division [81,84,85]. Stochasticity generates cell individuality independent on environmental cues and increases the likelihood of survival in fluctuating environments [81]. Phenotypic heterogeneity can also be a deterministic switch induced in response to stress exposure.

4.2 Environmental stress tolerance

The ability of microorganisms to survive fluctuating and stressful conditions depends on rapid changes in gene expression. Stress response in yeast is controlled by stress-activated protein kinase (SAPK) pathways that regulate transcription factors, kinases, cell cycle regulators and membrane proteins for adaptation of the internal milieu to the environmental conditions [86]. As a result, a common transcription response called the environmental stress response (ESR) is induced in yeast that entails inhibition of protein synthesis and activation of energy storage catabolism. Consequently, the cell enters a low metabolism quiescent state with increased stress tolerance [87,88]. However, genes induced by response to acute stress are not essential for surviving that stress, but rather prepare the cell for future stimuli. The initial stress triggers the ESR that serves a protective role for subsequent stimuli, and the second stress induce genes that are condition-specific and essential for survival [89,90]. Evolutionary selection for microorganisms in fluctuating stressful environments imposes a trade-off between fast growth and high stress tolerance [91]. While homogeneous, fast growing populations have high fitness in constant environments, phenotypic population heterogeneity ensures population survival in changing environments [80]. The strong anti-correlation between growth rate and stress survival can be observed in planktonic stationary state cells and mature biofilm cells that both contain heterogeneous populations with low cellular metabolism and high stress tolerance levels [92].

4.3 Quiescence

Yeast cells can exit the cell cycle and enter a growth arrested state called quiescence. This state can be induced by exposure to stress and has been well characterized for nutrient starved cells. The growth arrest is temporary and can be reversed by the addition of nutrients.

It is the general assumption that once yeast cells pass a restriction point in the cell cycle G_1 phase called START, they are committed to pass through all cell cycle phases until they reach the restriction point again

[93]. The cell can pass START in the presence of a good quality carbon source, but fail to do so in nutrient poor environments. Instead, the G_1 cells enter the G_0 phase, which is a quiescent growth arrested state [94] (Figure 6). This assumption was recently challenged with the observation that yeast cells can enter a quiescent state in all cell cycle phases [95]. Nonetheless, quiescent cells are most frequently observed in the G_1 phase, which might be a result of extended G_1 length when protein synthesis becomes limited in response to decreased nutrient availability rather than a cell cycle controlled event [95].

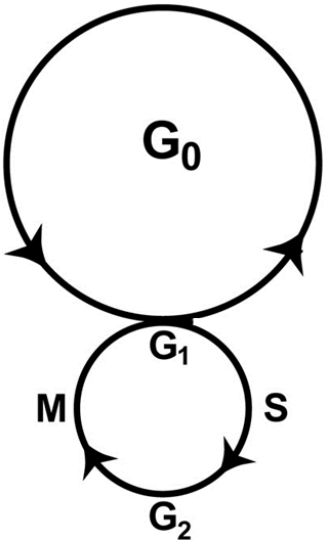

Figure 6. Quiescence. Yeast cells can in response to nutrient limitation exit the mitotic cell cycle and enter a quiescent state of growth arrest in the G_1 derived G_0 phase. When conditions become favorable for growth, the cells reenter the cell cycle. Figure modified from Allen *et al.* (2011) [96].

Quiescent cells are characterized by their ability to maintain viability in a growth arrested state for extended periods of time. The overall metabolism of the cell is reduced as a consequence of inhibition of translation initiation [97], reduced expression of ribosomal proteins [98,99], and repressed transcription by RNA polymerase II [100]. The quiescent cell state induces autophagy, a process by which the cell degrades non-essential cellular components to liberate metabolites under starvation conditions [94,101]. They also accumulate the carbohydrate trehalose for energy reserves [102] and increase fatty acid oxidation [103]. Furthermore, quiescent cells have a thickened cell wall and are tolerant to a variety of environmental stresses [94].

It has previously been assumed that all cells in the stationary growth phase were in a quiescent state [99]. However, it has later become apparent that stationary phase populations are likely to contain a mix of quiescent and non-quiescent cells [96]. Quiescent cells are dense, unbudded daughter cells formed after the diauxic shift. They can survive prolonged starvation and synchronously reenters the mitotic cell cycle.

Because quiescence can be entered in all cell cycle phases and transcriptome and metabolome profiles are specific for the inducing stimuli, quiescence is not a unique state [104,105]. Heterogeneous quiescent states might also be influenced by time-spent in growth arrest [104]. Non-quiescent cells are a heterogeneous population of replicative older cells that have lost the ability to reproduce and increase apoptosis during starvation [96,106].

Quiescent cells are poised for immediate response and rapid resumption of proliferation when conditions become favorable. RNA polymerase II is prepared to activate the transcription machinery by being located upstream of several genes that are inactive during quiescence, but are required for cell proliferation [99]. Furthermore, actin bodies and long term polarity cues are located at one cell pole, which ensure efficient initiation of polarized cell growth [107,108]. Re-suspending quiescent cells in nutrient rich media stimulate exit from quiescence and entry to the mitotic cell cycle after activation of protein synthesis [94]. Addition of metabolites like glucose activate mobilization of actin bodies and have been shown to be sufficient to trigger exit from quiescence without inducing growth [95]. Vacuolar membrane associated proteins belonging to the EGO complex ensure proper exit from G_0 [109] possibly by activation of the TORC1 pathway [110].

4.4 Nutrient sensing and growth control

TOR pathway

Cell growth control in response to nutrient availability is regulated by Target of Rapamycin (TOR). TOR is a highly conserved protein kinase belonging to the Ser/Thr family that in yeast forms two protein complexes called TOR complex 1 (TORC1) and TOR complex 2 (TORC2).

TORC1 is primarily located at the vacuolar membrane [111]. When nutrients are abundant, TORC1 positively controls ribosome biogenesis by activation of translation initiation factors, ribosomal protein transcription factors and RNA polymerases I, II and III [111,112]. The downstream effect of TORC1 activation is protein synthesis that facilitates cell proliferation (Figure 7). Additionally, TORC1 regulate cell cycle transition by activation of cyclin kinases [111]. In addition to promoting anabolic processes, TORC1 also stimulates growth by inhibiting catabolic processes associated with stress survival. Stress response programs in yeast, like the ESR, autophagy and the cell wall integrity pathway, have an inhibitory effect on growth, and are therefore suppressed by TORC1 in benign environments to ensure optimal growth [101,111]. TORC1 also regulates localization and expression of nutrient permeases for utilization of preferred nutrients sources before non-preferred [111].

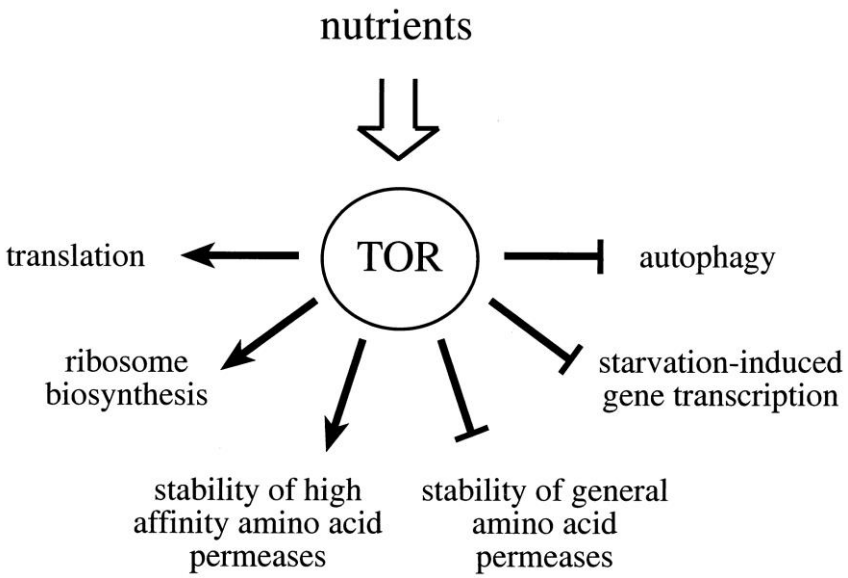

Figure 7. TOR. The TOR protein complexes control the balance between cell growth and stress survival. In the presence of good quality nutrient sources, TOR activates protein synthesis and inhibits transcriptional stress responses and autophagy. Nutrient starvation blocks TOR and results in growth arrest and induced stress response. Figure adapted from Raught *et al.* (2001) [113].

When the cell is starved for glucose or nitrogen, TORC1 becomes inhibited and the cell arrests growth. A similar response can be observed when the cell is treated with the immunosuppressive drug rapamycin [114]. Rapamycin form a complex with the protein FKB12 that inhibit the activity of TORC1 [115] and rapamycin treatment is responsible for the extensive knowledge available for TORC1 function.

TORC2 is insensitive to rapamycin and because no TORC2-specific inhibitor has been identified, much less is known about this protein complex. TORC2 is located at the plasma membrane and regulates cell polarity and endocytosis. TORC2 controls cell growth by actin cytoskeleton mediated supply of proteins and lipids to the proliferating daughter cell [101,111]. Additionally, TORC2 is involved in lipid homeostasis and is a positive regulator of sphingolipid biosynthesis [111,116].

PKA and SNF1

Nutrient sensing in yeast is very complex with multiple overlapping nutrient sensing pathways [101,117]. *S. cerevisiae* has besides TOR two other major nutrient sensing kinases that respond to nutrient availability, namely Protein Kinase A (PKA) and SNF1. PKA regulates cell growth in response to glucose and is required for the cell to execute START in the cell cycle while inactivation of PKA results in cell cycle arrest [117].

The SNF1 network regulates genes required for growth on non-preferred fermentable and non-fermentable carbon sources, and SNF1 is therefore required to achieve a stable growth arrested state [94].

5. Future treatment options

The major concerns in the hospital sector regarding fungal infections are the increasing numbers of patients susceptible to fungal infections, the more frequent isolation of azole resistant *Candida* species and the poor activity of conventional systemic antifungal agents against fungal biofilms. Although several second-generation triazoles and new echinocandins recently have been developed, new drugs with novel modes of action are limited for antifungal therapy (Figure 8). There is a demand for development of novel antifungal therapies, but the high similarity between fungi and humans limits the number of unique antifungal targets and can cause severe toxic side effects [44].

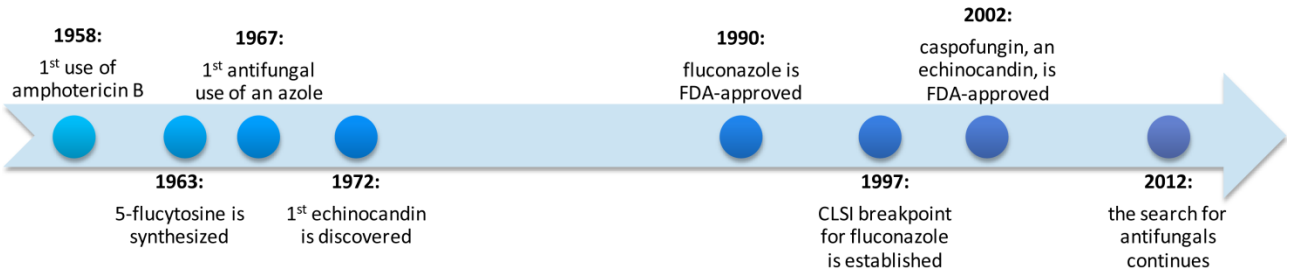

Figure 8. Antifungal drug development. No new major antifungal drug class that later has been introduced to clinical practice has been identified since 1972. Figure adapted from Butts and Krysan (2012) [118].

One approach to discover novel antifungal agents is to screen libraries of natural products or synthetic chemical compounds for antifungal activity [118]. Accordingly, natural oils with *Candida* inhibitory activity and anti-biofilm activity have been identified with monotherapy potential [119-121]. Synergistic activity between conventional antifungal agents and unrelated compounds has shown the possibility of new drug combination therapies against yeast biofilms [122-124]. Targeting virulence mechanisms such as biofilm formation is an alternative to inhibit essential cellular processes. Biofilm prevention and biofilm dispersion are suggested targets that can be achieved by use of prophylactic treatment, blocking QS signaling or dissolving extracellular matrix [73,125]. Biofilms are known for the existence of slow growing or growth arrested cells that can tolerate high concentrations of antimicrobial agents. Treatment targeting genes responsible for this phenotype might cause eradication of the entire cell population if it is combined with conventional antifungal agents [126,127]. Alternatively, stimulating exit from quiescence and increase metabolic processes that facilitate antifungal drug activity without inducing cell growth might be a future treatment strategy [95,128,129].

5.1 Antimicrobial peptides

Antimicrobial peptides (AMPs) have attracted a lot of attention in the past decades as a source for future drug discovery. AMPs are generally described as small (12-50 amino acids), cationic and amphipathic peptides that are part of the innate immune system of plant and animal species [130,131]. The physiochemical properties of AMPs facilitate membrane permeability and rapid cell lysis, but they can also target intracellular processes by inhibiting synthesis of DNA and proteins [132]. Several AMPs have a broad spectrum of activity and targets both bacteria and fungi, hence AMPs that exclusively targets fungi are less common than those with broad antimicrobial properties [131]. The commercial most successful antifungal peptides are members of the echinocandin family that presently are the only peptides with FDA approved use against systemic mycosis [131]. Other antifungal peptides include plant defensins and histatins but most of them have poor pharmacokinetic or pharmacodynamic properties [130,131]. The advantage of AMPs as therapeutic drugs is their novel mechanisms of action that make them active against strains that are resistant to the commonly used antifungals. It has also been suggested that the rapid killing kinetics and unspecific mechanism of action decrease resistance development, although resistance to AMPs have been observed [133]. The membrane permeabilizing AMPs are attractive candidates for anti-biofilm therapy because they can kill slow growing and growth arrested cells [130]. The disadvantages of naturally occurring peptides are loss of activity *in vivo* due to the presence of biological body fluids and proteolytic degradation, toxic side effects, rapid clearance and high production cost [130].

The drawbacks of natural AMPs can be overcome by chemical alterations of backbone and side chains that mimics the structure and function of natural peptides. Recent advances in organic chemistry have led to the development of systematic tools that, guided by rational design and molecular modeling, create a platform for future drug discovery. Because of inexpensive oligomers for *de novo* synthesis, peptidomimetics with protease stability can be produced at low cost [134,135]. Based on structure-activity relationship, antifungal activity and bioavailability can be optimized for therapeutic use [135].

6. *Saccharomyces cerevisiae* as a model organism

Saccharomyces cerevisiae (*S. cerevisiae*) is a species of yeast that is also known as baker's yeast or budding yeast. The fermentation properties of *S. cerevisiae* have been used for baking, beer and wine production since ancient time [136,137] and it is today one of the most extensively studied organisms. *S. cerevisiae* has a genome size of 12Mb with ~6000 genes (~1000 essential [138]) organized on 16 chromosomes [139]. It can be haploid or diploid containing one or two homologous copies of each chromosome, respectively. *S. cerevisiae* has a cell size of 5-10 µm and reproduces by mitosis, which is an asexual division where the daughter cell buds out from the mother [140]. Optimal growth conditions are anaerobically in rich medium with a carbon source, preferable hexoses like glucose and fructose, at 25-30°C, but it can grow at up to 42°C [141]. Natural habitats of *S. cerevisiae* are sugar-rich fruits such as grapes, peaches and oranges [142-144]. Because *S. cerevisiae* is not airborne [145] and lack motility proteins similar to flagella and pili in bacteria, it is being relocated by insects [146].

S. cerevisiae is a widely used model organism for several reasons. Besides its relatively high homology to human cells [147] and pathogenic yeasts [148], it is genetically tractable with high efficiency of homologous recombination and DNA transformation [149,150] allowing easy and cheap genetic manipulations [147]. Furthermore, *S. cerevisiae* has a fast growth rate and it was the first eukaryotic genome that was sequenced [139]. This has made *S. cerevisiae* the favored eukaryotic organism for the application of novel methods such as transcriptomics [151] and proteomics [152]. Since the 1990s, several genetic and bioinformatic tools have been developed in yeast for the study of biological functions of genes and how they interact [153]. Gene interactions and genotype to phenotype correlations can experimentally be studied in *S. cerevisiae* using methods such as synthetic genetic array (SGA) [154] and multiplexed barcode sequencing (Bar-seq) [155].

6.1 *S. cerevisiae* biofilm

While flocculation of *S. cerevisiae* is recognized as an important phenotype in the brewing industry that facilitate easy separation of yeast cells from the brew [156], it is not a desired trait in most microbiology laboratories because the flocculating lifestyle interferes with common cultivation and genetic assays. It is, however, important in the study of biofilm formation. Although *S. cerevisiae* was suggested as a model organism to study yeast biofilms more than a decade ago [3] not much progress have been made to develop and explore *S. cerevisiae* biofilm abilities and take advantage of all the molecular and genetic tools available for this organism compared to the pathogenic yeasts. Interestingly, *C. glabrata* is phylogenetically more closely related to *S. cerevisiae* than to other *Candida* species [157] and it has only been isolated as haploids with yeast morphology, which is in contrast to filamentation associated with *C. albicans* virulence

[158]. It has furthermore been shown that *S. cerevisiae* and *C. glabrata* have homologs in environmental stress response [159] and *S. cerevisiae* might therefore be a particularly good model for *C. glabrata* biofilms. Most importantly, *S. cerevisiae* possesses all the common traits that are associated with biofilm formation by *Candida* species.

Cell-surface adhesion

Attachment of *S. cerevisiae* cells to foreign surfaces is dependent on the cell surface protein Flo11 and deletion mutants are unable to from a biofilm [3]. *FLO11* is a homolog to the *ALS* and *EPA* gene family adhesins in *Candida* species [160]. Expression of *FLO11* is regulated by a complex network involving several major pathways in *S. cerevisiae* metabolism [161]. Stressful conditions such as starvation from glucose, nitrogen or amino acids is known to induce *FLO11* expression [156,161]. The *FLO11* gene is also responsible for other phenotypes in yeast that resembles biofilm formation, such as flocculation [162], invasive growth [163], pseudohyphae [164] and flor development [16]. Although *S. cerevisiae* have four other *FLO* adhesins (*FLO1, FLO5, FLO9* and *FLO10*) most laboratory strains only express *FLO11* while the others remain transcriptionally silent [165,166].

Extracellular matrix

S. cerevisiae cells growing as flocculating multicellular consortia in liquid media or as colonies on solid agar have by electron microscopy been shown to produce an extracellular matrix that encapsulate the cells. [167-170]. The extracellular matrix can be visualized as a dense layer around the cell wall, but is not part of the cell wall itself [168]. The extracellular matrix has been found to contain polysaccharides, mainly hexoses, and monosaccharides such as glucose and mannose [168]. Proteins unrelated to flocculins have also been isolated [167]. Production of an extracellular matrix in *S. cerevisiae* is dependent on the expression of *FLO* genes, but it is not essential for flocculation [168].

Quorum sensing

S. cerevisiae uses the QS molecules tryptophol and phenylethanol to activate *FLO11* expression [171] and they are therefore likely to influence *S. cerervisiae* biofilm development. Tryptophol and phenylethanol are aromatic alcohols that are structurally related to the *Candida albicans* QS molecule tyrosol [172].

Antifungal tolerance

Activity of clinical relevant antifungals against *S. cerevisiae* is not well studied probably because *S. cerevisiae* is not a pathogen. Therefore, the research on antifungal tolerance of biofilms formed by baker's

yeast is also limited. However, *S. cerevisiae* cells in a biofilm have been found to decrease susceptibility to disinfectant biocides [173] and antifungals [174], suggesting that *S. cerevisiae* biofilms have the common traits of stress tolerance that are observed in pathogenic yeasts.

7. Present investigations

Yeast biofilm cells are accredited for tolerating antimicrobial agents at concentrations that are up to 1000-fold higher than their planktonic counterparts [175]. Several studies have observed this by comparing minimal inhibitory concentrations (MIC) of planktonic cells with drug susceptibility of mature (48 hours) biofilms [17,78,174,176]. Based on this, tolerance is suggested to be biofilm-specific. One major concern when comparing the two susceptibility assays is the incubation time before the cells are exposed to antifungal agents. *In vitro* standard MIC assay of planktonic cells are defined by the use of fresh cultures with low cell densities and fast growth [177]. Antifungal susceptibility against biofilm cells often involves 48 hour cultures with high cell densities and slow growth [178]. By examining drug susceptibility under such distinct cultivation conditions, it can be expected that factors such as cell density, cell age and growth rate that are all unrelated to biofilm mode of growth *per se*, will mask true biofilm-specific tolerance mechanisms. These are all factors that are well described to also influence stress survival of planktonic cells [63,80,91,179] and can therefore be considered as artifacts of incubation time. It might be that the incubation time shapes the developing biofilm, but so will 48 hours growth of planktonic cells that in this time span have passed the diauxic shift and are about to enter stationary phase in nutrient rich media [180]. A 1000-fold increased tolerance to antimicrobial agents has also been reported for bacteria when comparing biofilm to planktonic cells. However, bacterial populations of biofilm and planktonic cells show similar tolerance to antimicrobial treatment when they have been cultivated under similar conditions, suggesting that planktonic cells can be just as tolerant to antimicrobials as biofilm cells [181-184]. To date, it has not been investigated if this also applies for fungal cells. In the experimental work presented in this thesis we cultivated biofilm and planktonic cells similarly to identify true yeast biofilm-specific tolerance mechanisms. The only difference was that the biofilm cells were grown on plastic surfaces to promote biofilm growth [3], while planktonic cells were grown in glass tubes that are incompetent for biofilm growth [185].

In the second part of this thesis we aimed to investigate the antifungal mechanism of action of a novel peptidomimetic called LTX-109. This drug has therapeutic potential as an alternative to the conventional antifungals in treatment of biofilm cells. LTX-109 was developed by the Norwegian pharmaceutical company Lytix Biopharma and it is currently in clinical phase II trials for topical treatment of bacteria. LTX-109 is a tripeptide based on the pharmacophore of bovine lactoferricin. It contains a modified tryptophan residue (tertbutyl tryptophan) and is capped by an ethylphenyl group at the C-terminal [186]. The small molecule size of LTX-109 and synthetic modifications makes it tolerant to proteolytic cleavage and cheap to produce [186,187]. LTX-109 disrupts the bacterial cell membrane and has low toxicity against mammalian

cells [186]. Furthermore, LTX-109 has activity against multiresistant bacterial strains [188] and derivatives have shown anti-biofilms activity [189].

We have in this thesis used the genetic tractable *S. cerevisiae* as a model organism for the pathogenic *Candida* species. A major advantage of using *S. cerevisiae* as a model system is the molecular tools developed for this organism, such as the barcoded gene deletion collection. Soon after the *S. cerevisiae* genome was published, a collaboration of European and North American laboratories finished the construction of a mutant library with deletions in 96% of all non-essential genes of *S. cerevisiae* [138,190]. A two-step PCR strategy that takes advantage of the high rate of homologous recombination in yeast was used to switch the gene of interest with a kanamycin resistance gene cassette (KanMX). Twenty-base oligomers with unique sequences for each mutant were simultaneously introduced up- and downstream the KanMX cassette equipping each mutant with molecular barcodes that can identify individual mutants in a pooled collection [138,190]. Together with the advances of next-generation sequencing, the mutant collection gives the opportunity to investigate thousands of strains simultaneously by screening the mixed population. Using multiplexed PCR strategies, the costs and labor can be reduced by amplification of barcode tags and incorporation of unique index sequences to each sample [155,191]. The gene deletions with associated barcodes can today be found in several strains and they were recently transferred to a biofilm competent strain [15]. This deletion collection provides a powerful tool to investigate the molecular basis for fungal biofilm development and drug tolerance.

7.1 Aim of study

The major objective of this thesis has been to investigate the multidrug tolerance mechanisms of yeast biofilm cells using *S. cerevisiae* as a model organism. Major emphasis has been on identifying true biofilm-specific tolerance mechanisms that are a result of the biofilm mode of growth and not artifacts of extended incubation time. Furthermore, we investigated the mechanism of action of a novel antifungal peptidomimetic to identify a new target, which potentially could be used in future antifungal therapy.

7.2 Overview of results

This section provides a short summery of the results obtained during the work on this thesis. A more comprehensive description of methods, results and figures can be found in the research papers listed in section 10.

Paper 1| Yeast biofilm tolerance towards systemic antifungals depends on growth phase.

In the first paper, we examined the susceptibility of yeast biofilm cells towards antifungal agents representing each of the four major drug classes currently used in systemic treatment of mycosis. This allowed us to investigate multidrug tolerance that covered the entire spectrum of clinically available drugs. Our results showed that flucytosine, the azole voriconazole, and the echinocandin caspofungin were inactive against 48 hours old biofilm for both *C. glabrata* and *S. cerevisiae*, while the polyene amphotericin B was the only tested drug with anti-biofilm activity. The results were compared to planktonic *S. cerevisiae* cells grown under similar conditions to investigate if the high tolerance levels were a unique trait for biofilm cells. We found that free-floating wild type cells and biofilm deficient *flo11* knockout mutants cultivated for 48 hours before drug exposure achieved the same level of antifungal tolerance as mature biofilm cells. These data showed that the observed antifungal tolerance was independent of planktonic or biofilm mode of growth.

A high proportion of the biofilm cells had decreased metabolic activity and limited growth after 48 hours in our biofilm model system, which confirms previous studies [192-195]. In order to obtain comprehensive knowledge on how cell proliferation affects the activity of each antifungal agent, killing kinetics of each drug were investigated in exponential and growth arrested planktonic populations. We found that *S. cerevisiae* cells could grow in the presence of voriconazole, which can be attributed to a process known as trailing. Flucytosine was found to be fungistatic, caspofungin killed the majority of yeast cells and amphotericin B was fungicidal against exponential growing *S. cerevisiae*. However, only amphotericin B had antifungal activity against growth arrested cells similar to what was observed in the mature biofilms. These results showed that antifungal activity of the tested drugs was highly dependent on growth phase.

Finally, we investigated the antifungal activity against proliferating biofilm cells and observed antifungal activity of all tested drugs similar to the killing kinetics study of exponential phase planktonic cells. These results demonstrated a strong correlation between growth activity of biofilm cells and drug treatment efficacy. The results were not surprising though, considering that fungistatic drugs and caspofungin targets biosynthetic pathways that are most active during cell growth and they will therefore not be expected to have activity against growth arrested cells in mature biofilms.

Paper 2| Cellular state determines amphotericin B tolerance in yeast biofilm and planktonic populations.

Slow growth was one of the first suggested tolerance mechanisms of biofilm cells [196] and, although a lot of research has been performed to discover additional tolerance mechanisms, it may still be the most pronounced contributor to antifungal recalcitrance. In paper 1 we showed that amphotericin B killed growth arrested cells, but was unable to eradicate the entire population of mature biofilm cells. So even

though slow growth seems like an appealing and simple explanation, it is insufficient to cover the amphotericin B tolerant subpopulation of mature biofilm cells.

To characterize the molecular mechanisms underlying amphotericin B tolerance, we performed a screen of a pooled collection of deletion mutants cultivated as biofilm or planktonic cells treated with amphotericin B. Surviving cells were outgrown to enrich for viable cells and genomic DNA was purified. Molecular barcodes that uniquely identifies each mutant were amplified and sequenced. We found that errors in four major biological processes could result in increased amphotericin B survival. Not surprisingly, defects in lipid metabolism increased amphotericin B survival probably because of decreased drug-target affinity caused by cytoplasmic membrane alterations. We furthermore found that gene deletions in intracellular transport, protein synthesis and cell cycle processes resulted in increased viability after amphotericin B treatment. Because protein synthesis and cell proliferation is controlled by the TOR pathway [101], we inhibited TORC1 by rapamycin treatment of wild type cells and observed that it caused a ~500-fold increase in amphotericin B survival compared to rapamycin untreated cells. These data suggested that the amphotericin B tolerant subpopulation was in a quiescent state that was physiological distinct from the sensitive subpopulation.

Our results showed that defects in the same biological processes resulted in increased survival to amphotericin B treatment regardless of cells were cultivated as biofilm or planktonically. This strongly suggested that it was not the biofilm mode of growth *per se* that caused antifungal drug tolerance, but rather it was a result of the physiological state of the cell that can be induced in both modes of growth.

Paper 3| The synthetic amphipathic peptidomimetic LTX109 is a potent fungicide that disturbs plasma membrane integrity in a sphingolipid dependent manner.

It can be a major challenge to target all heterogeneous subpopulations that are expected to be present in biofilms because of the limited number of antifungals with unique mechanisms of action. There is therefore a need for development of new fungicides with novel mechanism of action that can be used to supplement the commonly used antifungals.

In paper 3 we sought to describe the mechanism of action of the novel peptidomimetic LTX-109. We found that LTX-109 rapidly killed exponential growing *S. cerevisiae* cells, which was associated with an almost immediate release of protons, potassium and amino acids to the extracellular environment. We furthermore observed that LTX-109 treated cells readily took up a 600-Dalton fluorescent reporter molecule that only penetrates cytoplasmic membrane compromised cells. These data strongly suggested that LTX-109 targeted the plasma membrane and caused rapid cell lysis, rather than inhibited an intracellular pathway. To identify a potential molecular target of LTX-109, we screened a mixed population of yeast deletion mutants on agar plates containing the drug and sequenced the barcode tags of resistant

mutants. We found that deletion of genes in the sphingolipid biosynthesis pathway resulted in LTX-109 resistance, demonstrating an essential function of complex sphingolipids in LTX-109 fungicidal activity. These results indicated that LTX-109 destabilized the plasma membrane through direct or indirect interaction with the sphingolipids. We furthermore illustrated the potential use of LTX-109 for treatment of biofilm associated infection by showing that LTX-109 had anti-biofilm activity.

8. Conclusions and perspectives

Knowledge about the molecular mechanisms underlying tolerance of yeast biofilm cells to antifungal agents is important for development of novel treatment strategies. Multidrug tolerance of yeast biofilms does not seem to be solely mediated by individual mechanisms. As such, increased efflux pump activity only facilitates transport of azoles, while the other drugs are not substrates to any known efflux pumps. It has furthermore been shown that the antifungal agents can penetrate the extracellular matrix layer that encapsulates biofilm cells and it is the general consensus that the matrix layer does not contribute significantly to long-term antimicrobial treatment [73]. The close proximity of bacterial cells in biofilms have been suggested to facilitate a high rate of horizontal transfer of resistance genes [73], but acquisition of external DNA does not occur in intact yeast cells [57]. Finally, it has been observed that bacterial biofilm cells have an increased mutation rate that can result in development of antimicrobial resistance [73], but, except for flucytosine, antifungal resistance is a rare event in pathogenic fungi. Cross-resistance to multiple drugs is even more unlikely, because there are no multidrug resistance mechanisms in yeast that apply to all systemic drug classes. Cross-resistance should then evolve as a result of two independent mutations in two specific and unrelated genes with the possible exception of azoles and polyenes that are both dependent on ergosterols. Antifungal recalcitrance of biofilm cells are most likely mediated by non-inherited mechanisms as a result of physiological population heterogeneity.

We showed in paper 1 how antifungal drug activity was correlated with growth phase by testing the inhibitory efficacy against yeast populations with mainly growth arrested cells and against populations with mainly proliferating cells. A real life biofilm might consist of both growing and non-growing cells, but as long as they contain non-growing cells, the majority of antifungal agents currently used in the clinic are expected to be inactive against this subpopulation. Despite the possible influence of other factors, slow or no growth can alone explain the poor antifungal activity of most antifungal drug classes against biofilm cells. In addition to growth arrest, we found in paper 2 that defects in protein synthesis, intracellular transport, mitotic cell cycle and lipid metabolism resulted in increased survival of cells exposed to amphotericin B. This could be the result of stochastic events that caused fluctuations and variability in cellular processes such as DNA replication, cell membrane potential, ribosome activity and cytokinesis. We furthermore found that amphotericin B survival could be enhanced by nutrient starvation or inhibition of nutrient sensing pathways.

We have shown that planktonic cells can achieve a tolerance level similar to biofilm cells if they were cultivated in the same medium and for equally long time. We furthermore demonstrated that decreased metabolism, reduced growth rate and defects in the same biological processes were shared tolerance mechanisms between the two modes of growth. We conclude that antifungal tolerance is the combined

result of the mode of action of the antifungal agents and the physiological state of the cell. We suggest that growth in a nutrient limited environment for 48 hours induce a stress response resulting in acquired antifungal drug tolerance and that this can explain the enhanced tolerance levels observed in biofilm cells.

Due to the difficulty in diagnosis of biofilm infections and the observation that biofilm and planktonic cells respond similarly to antifungal treatment, it can be suggested that development of novel treatment strategies should target growth arrested cells without distinguish between sessile and free-floating cells. One approach could be to stimulate exit from quiescence, which have been shown to be initiated by exposure to metabolites without resulting in cell proliferation [95]. This might restore the activity of the systemic drugs that are inactive against quiescent cells. Another approach is to develop novel antifungal agents that target growth arrested cells for monotherapy or in combination with the conventional systemic drugs. Targeting extracellular components such as cell wall synthesis or cytoplasmic membrane components are obvious targets for novel antifungal agents, because they are likely to have fungicidal properties and they are not expected to be substrate to multidrug transporters. In paper 3 we identified sphingolipids as a novel antifungal target. Several natural peptides also inhibit the sphingolipids [197] and they are promising targets for future drug development because they are highly conserved among fungi, but distinct in human cells. Finding specific treatment against biofilm cells might only be relevant for treatment of catheter-related infections to which the surface attached property of biofilm cells undoubtedly is a biofilm-specific trait.

The data presented in this thesis rely on the value of *S. cerevisiae* as a model organism for the pathogenic *Candida* species. *S. cerevisiae* as a model might be limited because of its non-pathogenic behavior and because it has evolved under environmental conditions that are different from the natural habitats of *Candida* species. The findings in this thesis should therefore be confirmed in pathogenic yeasts. Our results from paper 1 indicate that *S. cerevisiae* and *C. glabrata* biofilms have similar antifungal sensitivity, but a more thoroughly experimental comparison needs to be performed to verify this.

9. References

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10. Research papers

The research papers included in this thesis are enclosed in the following order:

Paper 1

Bojsen, R., Regenberg, B., and Folkesson, A. (2014). Yeast biofilm tolerance towards systemic antifungals depends on growth phase. *Manuscript submitted to J Antimicrob Chemother*

Paper 2

Bojsen, R., Regenberg, B., Gresham, D., and Folkesson, A. (2014). Cellular state determines amphotericin B tolerance in yeast biofilm and planktonic populations. *Manuscript in preparation*

Paper 3

Bojsen, R.*, Torbensen, R.*, Larsen, C. E., Folkesson, A., and Regenberg, B. (2013). The synthetic amphipathic peptidomimetic LTX109 is a potent fungicide that disturbs plasma membrane integrity in a sphingolipid dependent manner. *PLoS One, 8(7): e69483*

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Title: Yeast biofilm tolerance towards systemic antifungals depends on growth phase

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Running title: Yeast biofilm tolerance to antifungal agents

Keywords: yeast, biofilms, drug tolerance, antifungal agents, metabolism, stationary state, fungicides, cultured cells

Author contribution

Designed the experiments: RB BR AF

Performed the experiments: RB

Analyzed the data: RB BR AF

Wrote the paper: RB BR AF

ABSTRACT

Biofilm-forming *Candida* species cause persistent infections that are difficult to eradicate, possibly because of antifungal drug tolerance mechanisms specific to biofilms. We used *Saccharomyces cerevisiae* as a model for drug susceptibility of yeast biofilms. Confocal laser scanning microscopy showed that *S. cerevisiae* and *C. glabrata* form similarly structured biofilms and that the viable cell numbers were significantly reduced by treatment of mature biofilms with amphotericin B but not voriconazole, flucytosine, or caspofungin. We showed that metabolic activity in yeast biofilm cells decreased with time, as visualized by FUN-1 staining, and mature, 48-hour biofilms contained cells with slow metabolism and limited growth. Time-kill studies showed that in exponentially growing planktonic cells, voriconazole had limited antifungal activity, flucytosine was fungistatic, caspofungin killed the cells, and amphotericin B was fungicidal. In growth-arrested cells, only amphotericin B had antifungal activity. Confocal microscopy and colony count viability assays revealed that the response of growing biofilms to antifungal drugs was similar to the response of exponentially growing planktonic cells in time-kill studies and mature biofilm was similar to non-growing planktonic cells. These results confirmed the importance of growth phase on drug efficacy. In conclusion, we showed that *in vitro* susceptibility to antifungal drugs was independent of biofilm or planktonic growth mode. Instead, drug tolerance was a consequence of a physiological cell state achievable by both planktonic and biofilm populations. Based on our results, drug treatment strategies should target non-dividing cells rather than focusing on biofilm-specific tolerance mechanisms.

INTRODUCTION

Nosocomial fungal infections are an increasing problem for immune compromised patients with severe underlying disease[1]. Fungi can colonize mucosal surfaces in the oral cavity, airways, wounds and the gastrointestinal tract, causing chronic infections[2]. Fungi can also adhere to invasive medical devices and cause severe septicemia[3]. It is thought that many chronic human infections are associated with the formation of microbial biofilms[4]. The hallmarks of biofilms are surface attachment and production of an extracellular matrix (ECM)[5]. Failure to eradicate chronic infections is often attributed to the unique lifestyle of cells in biofilms and it is widely accepted that cells in a biofilm possess antimicrobial tolerance mechanisms that are distinct from their planktonic counterparts. However, in spite of decades of research, the molecular mechanisms responsible for biofilm-specific drug tolerance are not fully elucidated[6].

Drugs currently being used to treat systemic mycoses belong to four major classes. The azoles target cytochrome P450 and inhibit cell membrane ergosterol biosynthesis, resulting in accumulation of toxic ergosterol intermediates[7]. The echinocandins inhibit 1,3-β-glucan synthases, resulting in a reduction in cell wall 1,3-β-glucan[8], and the polyenes target ergosterol and cause pore formation in the fungal cell membrane[9]. The fourth class is the antimetabolite flucytosine. Flucytosine is deaminated upon uptake in susceptible cells and converted to 5-fluorouridine triphosphate, which is incorporated into RNA, inhibiting protein synthesis[10]. Flucytosine can also be converted to 5-fluorodeoxyuridine monophosphate which acts on thymidylate synthase to inhibit DNA synthesis[10]. Despite the pronounced diversity in antifungal mechanism of action, most antifungal agents are inactive against fungal biofilms.

Several mechanisms have been suggested to be responsible for drug tolerance of yeast biofilms, but none of them can solely account for the multidrug tolerance associated with biofilms. The contribution of the ECM to antifungal drug recalcitrance is not fully understood. Although some antifungal drugs bind to ECM components they can still diffuse through the matrix layer in inhibitory concentrations[11-13]. The ECM, in combination with the nutrient-limited environment that results from a large number of microbial cells, might induce expression of genes that help cells cope with stressful conditions. Altered gene expression could involve differential regulation of general stress-response genes that affect drug tolerance. For example, efflux pumps are reported to be upregulated in young[14] and intermediate[15] biofilms in *Candida* species. However, efflux pump knockout mutants remain drug resistant[14, 16] and up-regulation is lost in mature biofilms[14, 15]. Furthermore, since polyenes and echinocandins are not a substrate of any known efflux pumps[17], efflux pumps are not solely responsible for biofilm-mediated tolerance to these drug classes. Decreased levels of drug target molecules are reported in maturing

Candida spp. biofilms[14, 18], but not for all drug classes. It might be a combination of several individual mechanisms that cause multidrug tolerance in yeast biofilms. Biofilms consist of heterogeneous populations of phenotypically diverse cells. This heterogeneity is caused by the nonuniform and structured environment of the biofilm[19]. To be effective against biofilms, an antifungal drug must target all of the multiple subpopulations in the biofilm.

Fungal and bacterial research report 1000-fold higher tolerance level of mature biofilms compared to proliferating planktonic populations[20, 21]. However, research in bacteria has shown that the tolerance phenotype is similar between biofilm and planktonic cells when grown to stationary state [22-24]. This indicates that tolerance mechanisms are not biofilm-specific and that planktonic cells can achieve the same level of tolerance. To date, comparison between biofilms and planktonic cells cultivated under similar conditions has not been performed in yeast.

C. glabrata is the second most-frequent cause of candidemia[25] and is phylogenetically more closely related to *Saccharomyces cerevisiae* than to other *Candida* species[26]. The biofilm structure of *C. glabrata* is also more similar to *S. cerevisiae* than to *C. albicans. C. glabrata* form a thin layer of biofilm that contain haploid blastospores and lack hyphae and pseudohyphae morphology that are characteristic to *C. albicans* biofilms[27, 28]. Furthermore, *C. glabrata* has environmental stress response homologs to *S. cerevisiae*[29]. *S. cerevisiae* has all the traits commonly associated with microbial biofilms and is more easily manipulated than *C. glabrata*[30] making *S. cerevisiae* a relevant model for this pathogen. We used *in vitro* biofilms of *S. cerevisiae* and *C. glabrata* cultures to investigate antifungal tolerance to the major antifungal drug classes used for systemic treatment of human pathogenic fungal infections: the polyene amphotericin B (AmB), the azole voriconazole (VOR), the antimetabolite flucytosine (5FC), and the echinocandin caspofungin (CAS). We have in the present study performed a thoroughly study of the relation between the mechanism of action of the individual drugs and the drug tolerance level of yeast cells. To identify biofilm-specific mechanisms, free-floating planktonic cells were used for comparison. We found that the ability of biofilms to survive antifungal treatment was dependent on the mode of action of the antifungal agent and the metabolic state of the yeast cells.

MATERIALS AND METHODS

Yeast strains: *S. cerevisiae* Σ1278b YS-11 (*MAT***a** *can1Δ*::*STE2p-spHIS5 lyp1Δ*::*STE3p-LEU2 his3*::*HisG leu2Δ ura3Δ*) was used as reference strain (a gift from the Boone Laboratory, University of Toronto). A *flo11* mutant that does not form biofilm was obtained from the Σ1278b gene deletion library[31]. *C. glabrata* (ATCC 90030) was obtained from the American Type Culture Collection. A strain expressing green fluorescent protein (GFP) was constructed by expressing the *GFP* gene from the *TEF1* promoter. The *TEF1* promoter was PCR amplified from pSP-GM2[32] with primers 5'-CGTGCGAUGCCGCACACACCATAGCTTC and 5'-ACGTATCGCUGTGAGTCGTATTACGGATCCTTG. GFP was amplified from pJBA27a[33] with primers 5'-AGCGATACGUAGCATGCGTAAAGGAGAAGAA and 5'-CACGCGAUTATTTGTATAGTTCATCCATGCC. The *GFP* and *TEF1* DNA fragments were assembled with USER technology[34] and inserted into vector pXI-2[35] digested with *Asi*SI and nicking enzyme Nb.BsmI and transformed into competent *Escherichia coli* (DHα5) as previously described[36]. The P_{TEF1}-GFP fragment was inserted in chromosome XI position (91,575..92,744) of the reference strain using a high-efficiency transformation protocol[37] and selected on uracil depleted agar.

Media and antifungals: All experiments were performed in synthetic medium (0.67% yeast nitrogen base supplemented with glucose and amino acids)[38]. A 0.2% (w/v) glucose concentration was used in all biofilm experiments. Yeast extract peptone dextrose (YPD)[38] agar plates were used for colony counting. Antifungals voriconazole, flucytosine, amphotericin B and caspofungin were from Sigma-Aldrich. All antifungals were dissolved in DMSO in 5 mg/ml stock solutions and stored at -20°C. All experiments were performed in triplicate.

Minimal inhibitory concentration: Minimal inhibitory concentrations (MIC) were determined as previously described[39] with modifications. In short, two-fold dilution series of antifungal drugs were prepared in fresh synthetic medium with 2% glucose (w/v) and distributed into 96-well microtiter plate. Visibly turbid overnight cultures were diluted to OD_{600} 0.1 in fresh medium and transferred to microtiter plates containing aliquots of serially diluted antifungal drug. Plates were statically incubated at 30°C for 24 h and absorbance was measured with a microplate spectrophotometer (BioTek PowerWave 340). Growth inhibition of ≥ 50% was determined as MIC for CAS, VOR and 5FC and ≥ 90% growth inhibition was determined as MIC for AmB as recommended by EUCAST[39].

Visualization of biofilm drug susceptibility: Visibly turbid cultures were diluted to OD_{600} 0.1. After 2 hours at 30°C, cells were transferred to biofilm chambers (Technical University of Denmark) with a polyvinyl chloride (PVC) coverslip surface (Rinzl, Electron Microscopy Sciences). After 4 or 48 hours static incubation at 30°C, medium was removed from biofilm chambers and centrifuged and antifungal drug was added to the supernatants at 10 times the MIC. Spent medium with drug was introduced to biofilm cultures followed by 24 hours at 30°C. Chromosomally integrated GFP and 3 µM Syto9 (Invitrogen) were used to visualize live cells and 1 µM propidium iodide (PI, Sigma-Aldrich) was used to stain dead cells. Confocal laser scanning microscopy (CLSM) was performed with a Zeiss LSM710 microscope equipped with excitation lasers at 488 nm and 514 nm. Imaging used an EC Plan-Neofluar 40x/1.30 Oil lens.

Metabolic activity: Preparation of cell cultures and CLSM imaging was as described above except a Plan-Apochromat 63x/1.40 Oil DIC M27 objective was used. Metabolically active cells were distinguished from inactive cells with 10 µM FUN-1 as described by the manufacturer (Molecular Probes, Probes for yeast viability, MP 07009). Cells were considered metabolic active if they produced red cylindrical intravacuolar structures[40, 41].

Killing kinetics: Overnight cultures were diluted to OD_{600} 0.01 in fresh synthetic medium. Yeast cultures were grown to exponential phase in baffled shake flasks at 30°C and samples were distributed to test tubes for exposure to antifungal drugs at 5 times the MIC before incubation at 30°C with aeration. Samples were extracted at indicated time-points. Colony forming units (CFUs) were determined by plating serial dilutions on YPD agar.

Antifungal survival assay: Visibly turbid cultures were diluted to OD_{600} 0.1 in synthetic medium and grown in baffled shake flasks for 2 hours. Culture samples were distributed to glass tubes for planktonic cells and polystyrene microtiter plates for biofilms and incubated statically at 30°C. After 4 or 48 hours, cells were challenged with antifungal drug at 10 times the MIC, added in spent medium, for 24 hours. Viable cells were determined by counting CFUs on YPD agar. Biofilm cells were washed twice in saline and CFU was determined.

Statistical analysis: Unpaired Student's *t*-test was used for statistical analysis. $P < 0.01$ was considered significant.

RESULTS

S. cerevisiae and *C. glabrata* biofilms have similar structure and antifungal tolerance

Antifungal drug susceptibility of *S. cerevisiae* cells were similar to *C. glabrata* as determined by minimal inhibitory concentrations (MIC), except for voriconazole that was 4 mg/l against *C. glabrata*, and 1 mg/l against *S. cerevisiae* (Figure 1a).

Yeast biofilm architecture and antifungal drug sensitivity was investigated using CLSM. Mature GFP-tagged biofilm cells were challenged with an antifungal agent for 24 hours and stained with PI to mark dead cells. Untreated *S. cerevisiae* biofilms contained a thin layer of cells (approximately 30 µm) with a few dead cells distributed throughout the biofilm. Biofilms treated with VOR, 5FC, or CAS had the same architecture and mixture of living and dead cells as untreated control cells (Figure 1b), showing that the drugs were inactive against yeast biofilms. AmB was the only tested drug with anti-biofilm activity, killing most cells after 24 hours (Figure 1b). The small subpopulation of cells that survived AmB treatment was randomly distributed in the biofilms.

To determine if results from the *S. cerevisiae* biofilm model applied to drug susceptibility in a pathogenic yeast, we investigated the antifungal drug susceptibility of *C. glabrata* biofilms. *C. glabrata* was cultivated under conditions similar to *S. cerevisiae* cultures and developed a thin layer of biofilm cells (approximately 25 µm). After 48 hours, mature biofilms were challenged with an antifungal drug for 24 hours and stained with Syto9 and PI to visualize living and dead cells. Results were similar to those obtained with *S. cerevisiae*. Most *C. glabrata* biofilm cells exposed to VOR, 5FC or CAS showed living cells with a few dead cells distributed in the biofilm similar to the appearance of the untreated control cells (Figure 1b). AmB treatment killed most cells with a small, surviving subpopulation randomly distributed in the biofilm. These results suggested that *S. cerevisiae* could be used as a model organism to study antifungal tolerance in biofilms of the pathogenic *C. glabrata*.

Metabolic activity of biofilm cells decreases with biofilm maturity

Planktonic microbial cells cultivated in a closed system take up nutrients from the environment and enters a stationary growth state with decreased metabolic activity when nutrients become limited. To investigate if the metabolic activity of biofilms at 48 hours was reduced compared to a 4 hour biofilm, we measured metabolic activity using FUN-1 staining. FUN-1 permeabilizes the plasma membrane and biochemical processing of the dye by an unknown pathway identifies metabolically active cells with intravacuolar structures[40]. Most cells in a 4-hour biofilm showed high metabolic activity as estimated by staining intensity (Figure 2), but decreased with biofilm incubation time. After 48 hours, only a small

196 subpopulation of cells in the biofilms showed FUN-1 staining of vacuoles indicating a lower or different
197 metabolic activity in mature biofilm than that found in young 4 hour biofilm (Figure 2).
198
199 **Activity of antifungal drugs depends on growth state**
200 We hypothesized that the limited metabolic activity observed in cells in mature biofilms may be an
201 important cause of the low antifungal activity of the drugs tested. We measured therefore the killing
202 kinetics of the antifungals using an exponentially growing planktonic population and a growth-arrested
203 planktonic population. Untreated control cells proliferated with a doubling time of 1.5 hours in the
204 exponential growth phase for the first 8 hours of incubation (Figure 3a). The density of cells exposed to
205 VOR increased at the same rate as the untreated sample for the first 7 hours of incubation. Subsequently,
206 the azole drug inhibited growth, resulting in a decrease in viability after 24 hours. After two hours of
207 exposure to 5FC, the growth of exponential phase populations was inhibited and cells remained at the
208 same viability and density for 24 hours showing that 5FC had fungistatic activity. CAS had an inhibitory
209 effect on exponential growth within the first hours of exposure and a consistent killing rate throughout the
210 experiment that resulted in a 10-fold reduction in CFU after 24 hours compared to the initial population.
211 Challenging cultures with AmB rapidly decreased the viable population, reaching the lower detection
212 limit for CFUs after 5 hours.
213 We next investigated how growth arrest affect susceptibility to antifungal agents by incubating cells in
214 carbon-depleted medium. Starting cell density was similar to the starting density used for time-kill studies
215 on the exponential growing populations to eliminate cell numbers from affecting comparisons between
216 the two experiments. Growth-arrested *S. cerevisiae* exposed to VOR, 5FC or CAS had viability similar to
217 untreated control cells, showing that the drugs had no activity against non-growing cells in stationary
218 phase (Figure 3b). Cells exposed to AmB were killed, but the killing rate was lower than the rate observed
219 for exponentially growing cells. The lower detection limit for CFUs was not reached in the first 8 hours of
220 drug exposure, but only after 24 hours.
221
222 **Drug sensitivity restored in a growing biofilm population**
223 The antifungal activity of 5FC, CAS and AmB against exponentially growing planktonic *S. cerevisiae* but
224 not against growth-arrested cells suggested that drug activity depended on cell growth. To test if growth-
225 dependent drug activity also applied to cells in biofilms, the viability of a growing *S. cerevisiae* biofilm
226 was quantified as CFUs and visualized with CLSM. Quantification and visualization assays were
227 conducted after 24 hours of drug treatment. The number of cells in untreated 4-hour biofilms increased 8-
228 fold over 24 hours, as determined by CFUs (Figure 4). Growth in 4-hour biofilms exposed to VOR or
229 5FC was inhibited compared to untreated control biofilms. Cell numbers determined by CFUs increased

3-fold with VOR treatment and 1.3-fold with 5FC treatment. In contrast, 80% of biofilm cells exposed to CAS were killed, with surviving cells sporadically distributed in the biofilm (Figure 4). AmB had a fungicidal effect on young biofilms, killing 99.7% of cells during a 24-hour exposure. Biofilms treated with AmB still contained minor surviving subpopulations (Figure 4).

Mature biofilm and planktonic yeast have similar susceptibility to systemic antifungals

We observed that most drug classes tested in this study were inactive against mature biofilms (Figure 1), but had activity against 4-hours biofilms (Figure 4), and that this might be correlated to the metabolic state of the cell (Figure 2). These data suggest that the physiological state of the cell in response to ceased proliferation, rather than a biofilm-specific response mediate drug tolerance in yeast biofilms.

To determine the effect of biofilm formation on drug tolerance, we investigated differences in antifungal drug susceptibility between cells grown planktonically or in biofilms for 48 hours. *S. cerevisiae* biofilms were cultivated on flat polystyrene surfaces. For planktonic control populations, *S. cerevisiae* was cultivated in glass tubes[42]. An isogenic biofilm-deficient *flo11* knockout mutant was included as a negative control for biofilm formation[43].

All three cultures, biofilm, *flo11* control, and planktonic, were challenged for 24 hours with antifungal agents. No significant effects on CFUs were seen after treatment with VOR, 5FC, or CAS (Figure 5), indicating that *S. cerevisiae* was not susceptible to any of the drugs under any of the three growth conditions. Only treatment with AmB significantly decreased population sizes ($P < 0.01$, Student's *t*-test). Exposure to AmB killed 95-98% of the yeast populations regardless of growth condition (Figure 5). However, in all three growth conditions, a subpopulation of 2-5% of cells survived drug treatment, so AmB was unable to eradicate the entire *S. cerevisiae* population.

DISCUSSION

In the current study, we found that antifungal drug efficacy against yeast biofilm was dependent on cell growth. Only growing yeast cells were susceptible to growth inhibition by the fungistatic drugs VOR and 5FC, and killing by CAS. However, yeast cells in both growing and stationary state were efficiently killed by AmB. We further observed that the effects of antifungals were independent of biofilm or planktonic modes of growth.

Multidrug tolerance mechanisms in biofilms are suggested to include production of an ECM and a densely packed microbial structure that shields cells, preventing antimicrobials from reaching their targets. Cell-surface proteins in the Flo family are responsible for *S. cerevisiae* adhesion and ECM production is dependent on Flo expression. Flo11p is the only flocculation protein expressed in the Σ1278b *S. cerevisiae* strain and it is essential for biofilm formation[43-46]. We showed that a *flo11* mutant has an antifungal tolerance phenotype that is similar to mature yeast biofilms (Figure 5). This finding suggests that flocculation and Flo11p-dependent matrix production are not obstacles for cell penetration by antifungal drugs. In agreement with this, systemic antifungals penetrate the ECM of *Candida* species biofilms at concentrations that exceed the MIC values and no correlation is observed between the amount of matrix produced and drug susceptibility[11, 47]. These findings indicate that matrix and biofilm formation have less of an influence on drug susceptibility than previously thought. Nonetheless, surface attachment and matrix production might still contribute to persistent infections by reducing pathogenic cell clearance from body fluids and killing by immune cells[48, 49].

Heterogeneous microbial biofilms often contain large subpopulations with low metabolic activity[41, 50]. We showed that the metabolic activity of most cells in yeast biofilms decreased as the biofilm matured (Figure 2) and we observed no increase in cell density in mature biofilms (Figures 5a). Therefore, large fractions of cells in mature biofilms are likely in a stationary state that reduces the susceptibility to the antimicrobials. Even though antimicrobial agents have diverse modes of action, most are dependent on active growth to kill cells[51], which we confirmed in the present study.

The drug 5FC has fungistatic activity against *Candida* species[52]. We found a similar fungistatic activity against proliferating *S. cerevisiae* Σ1278b planktonic and biofilm populations (Figure 3a and Figure 4). However, 5FC was inactive against mature biofilms and growth arrested planktonic cells (Figure 1b, Figure 3b and Figure 5a). This result is not surprising since fungistatic drugs do not kill cells but only inhibit proliferation. Therefore, the viability of high-density, nongrowing microbial populations such as mature biofilms or stationary phase planktonic cells is expected to be unaffected by treatment with fungistatic drugs.

Echinocandins have fungicidal activity against *Candida* species[53] and we found that CAS killed 90% of exponential growing planktonic *S. cerevisiae* cells (Figure 3a) and 80% of proliferating biofilm cells (Figure 4). Despite the ability of CAS to kill exponentially growing yeast cells, CAS had no activity against mature biofilms (Figure 1b and Figure 5a). CAS inhibits 1,3-β-glucan synthase, which disrupt the yeast cell wall and result in osmotic stress and cell lysis[8]. However, the synthase is most active in growing cells[54, 55], so CAS is unable to kill growth-arrested cells[53] as we observed in this study (Figure 3b) including the cells in mature biofilms.

The polyene AmB was the only drug tested in this study with activity against cells in mature biofilms (Figure 1b and Figure 5a) and growth arrested planktonic cells (Figure 3b). AmB binds to ergosterol in the cell membrane and form pores that increase the permeability of electrolytes and small molecules. Pore formation results in loss of membrane potential and eventually cell lysis[9]. Since ion diffusion and lysis are passive processes, cell metabolism is not required for AmB to kill cells. Consistent with this mechanism, AmB killed both growing and non-growing yeast cells (Figure 3). However, although AmB killed cells in non-proliferating, low-density yeast populations, AmB-tolerant subpopulations were observed in stationary state planktonic and biofilm populations (Figure 5).

Azole drugs are fungistatic against *C. albicans*, but less active against other *Candida* species, which show a clear increase in cell density after azole treatment[56, 57]. The poor efficacy of the azole drug VOR against *S. cerevisiae* cells within the first 7 hours of exposure (Figure 3a) might be because *S. cerevisiae* and the closely related *C. glabrata* share a mechanism that makes them intrinsically resistant to azoles.

Bacterial cells grown as biofilms or grown to stationary state as planktonic cells have similar drug-tolerance phenotypes[23, 24, 58, 59]; we have in the present study extended this phenotypic similarity to yeast cells (Figure 5). Our results indicated that the biofilm mode of growth itself does not result in antifungal tolerance. Rather, the lack of cell division and the physiological state of stationary phase cells is responsible for the drug-tolerant phenotype.

A combination of factors is probably responsible for the multidrug tolerance of cells in biofilms. However, as long as biofilm populations contain non-proliferating cells, some of the most commonly used antimicrobials will have reduced efficacy. Biofilm tolerance to drugs is conditional and depends on the mode of action of the tested drugs, as well as cell physiology and environment[60]. We showed that standard laboratory yeast biofilm models and methods can determine cell culture conditions under which antifungal drugs are effective or ineffective. However, our models and methods did not identify biofilm-specific mechanisms of drug tolerance. Our findings imply that biofilm tolerance phenotypes might be caused by the large number of stationary cells within mature biofilms rather than specific biofilm mechanisms. Our data therefore suggest that future research on novel drugs and treatments should focus

on strategies that are effective against stationary non-growing cells, rather than attempting to develop specific anti-biofilm treatments.

ACKNOWLEDGMENT

This work was supported by the Danish Agency for Science Technology and Innovation is (FTP 10-084027).

We thank Charlie Boone and Owen Ryan (University of Toronto) for the Σ1278b strains. We also thank Tomas Strucko (Technical University of Denmark) for GFP construct strategy design.

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FIGURES

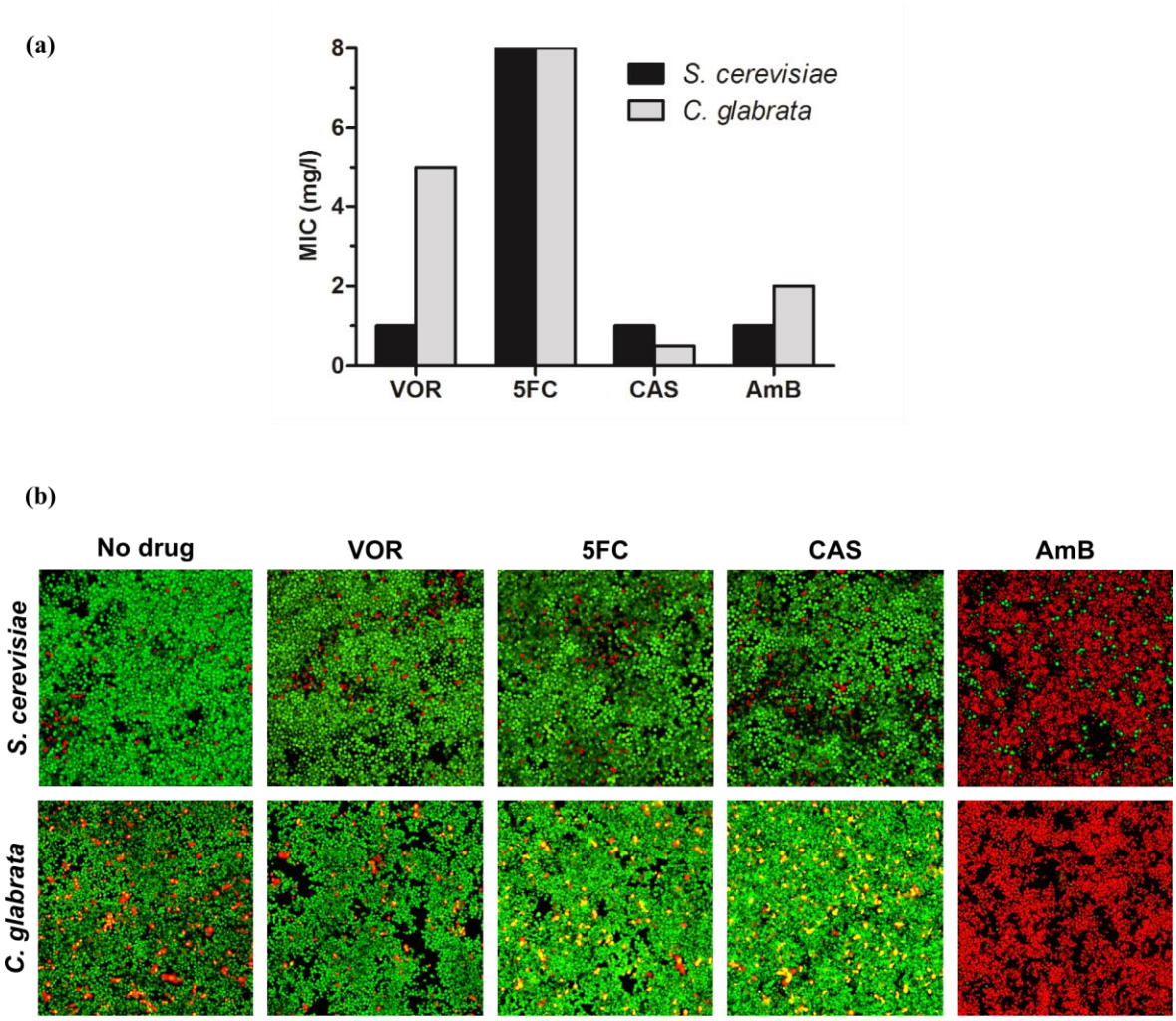


Figure 1. *Saccharomyces cerevisiae* and *Candida glabrata* biofilms have similar sensitivity to antifungal drugs. (**a**) MIC values of the indicated antifungal agents against *S. cerevisiae*. (**b**) Antifungal drug activity against 48-hour biofilms was visualized by confocal laser scanning microscopy. GFP-tagged *S. cerevisiae* was stained with propidium iodide (PI, 1 µM) to mark living (green) and dead (red) cells. *C. glabrata* was stained with Syto9 (3 µM) and PI (1 µM) to mark living (green) and dead (red/yellow) cells. Biofilm cells were treatment was for 24 hours with the indicated antifungal agents. VOR: voriconazole, 5FC: flucytosine, CAS: caspofungin, and AmB: amphotericin B.

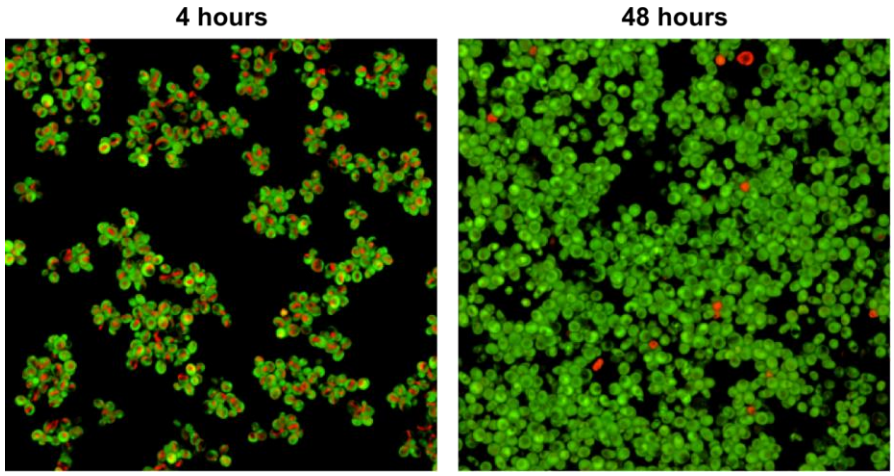


Figure 2. Metabolic activity of yeast cells in biofilms decreases with incubation time. *S. cerevisiae* cells stained with FUN-1 (10 µM) after incubation for 4 hours and 48 hours. Metabolically active cells produce red cylindrical intravacuolar structures.

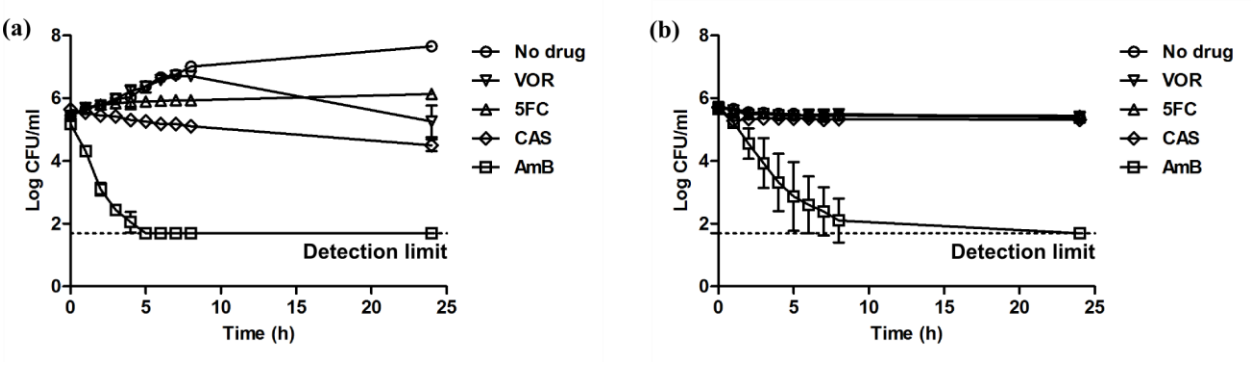


Figure 3. Amphotericin B is the only drug tested with activity against growth-arrested cells. (**a**) Killing kinetics of exponentially growing planktonic *S. cerevisiae* cultivated in 2% glucose exposed to 5 times the minimum inhibitory concentration (MIC) of the indicated antifungal drugs. (**b**) Killing kinetics of growth-arrested planktonic *S. cerevisiae* cultivated in carbon-depleted minimal media exposed to 5 times MIC of the indicated antifungal drugs. VOR: voriconazole, 5FC: flucytosine, CAS: caspofungin, AmB: amphotericin B. n = 3, error bars show standard deviations.

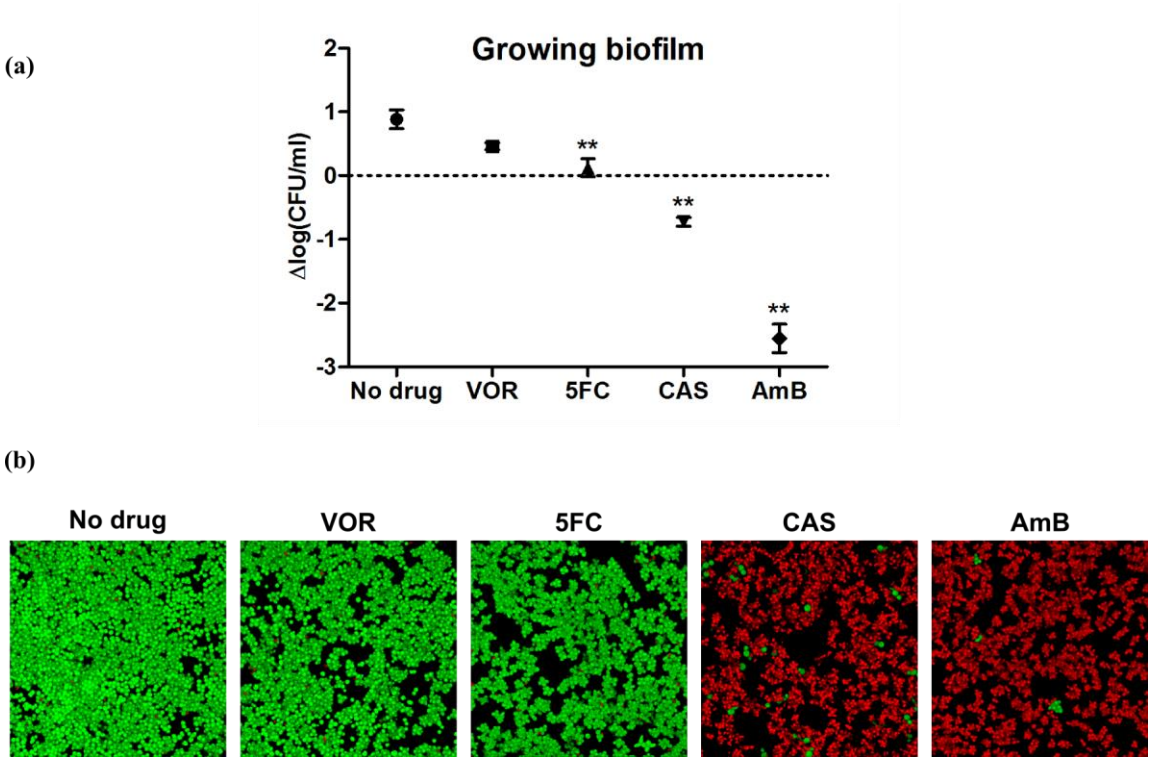
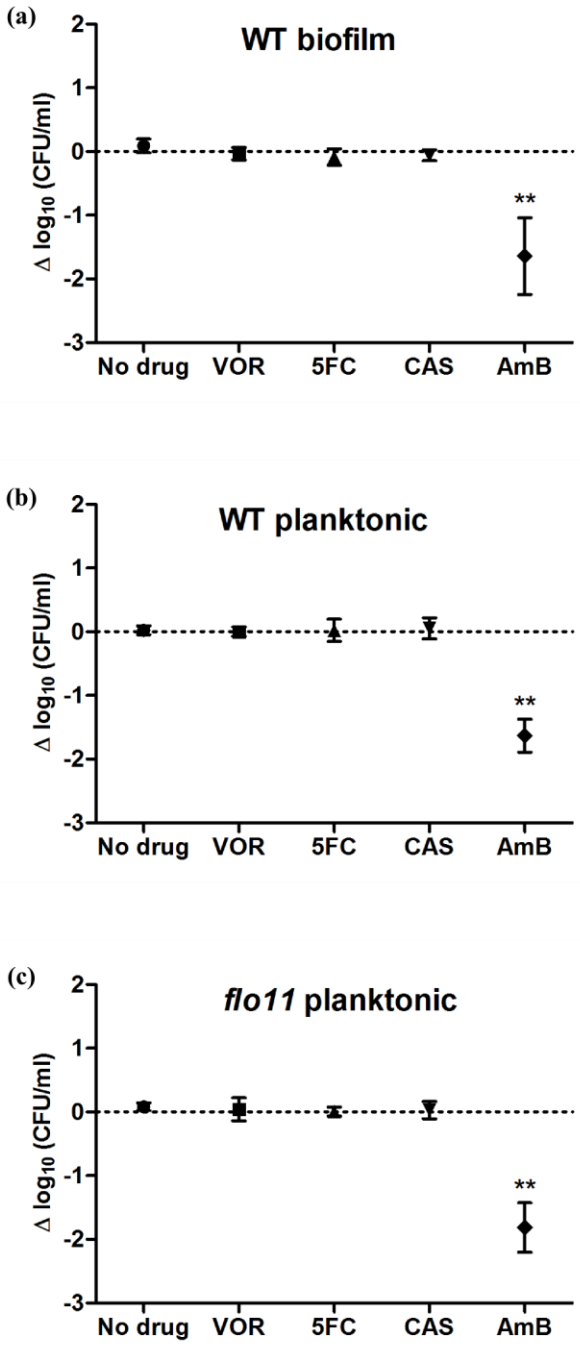


Figure 4. Antifungal drugs are active against growing biofilms. (**a**) Quantitation of biofilm cell viability. Biofilms were grown for 4 hours and viability determined as CFUs. Biofilms were then treated with 10 times the minimum inhibitory concentration (MIC) of the indicated antifungal drug for 24 hours and CFUs were measured. Shown is log change in CFUs. n = 3, error bars are standard deviations. Significance was evaluated with Student's t-test. **P < 0.01. (**b**) GFP-tagged S. cerevisiae in 4-hour biofilms stained with 1 μM propidium iodide visualized by confocal microscopy. Biofilm cells were treatment was for 24 hours with the indicated antifungal agents. VOR: voriconazole, 5FC: flucytosine, CAS: caspofungin, and AmB: amphotericin B.



494
495

496 **Figure 5.** Yeast cells grown in biofilms and planktonic cultures have similar drug tolerance. Cultures
497 were grown for 48 hours and viability was measured as colony-forming units (CFUs). Cells were treated
498 with 10 times minimum inhibitory concentration (MIC) of the indicated antifungal drug, or left untreated
499 as a control, and CFUs were measured after 24 hours. Shown is log change in viability. (**a**) Wild type *S.*
500 *cerevisiae* Σ1278b grown as biofilms on polystyrene surfaces. (**b**) Wild type grown planktonically in glass

flasks. (**c**) *flo11* knockout mutant grown planktonically in glass flasks. VOR: voriconazole, 5FC: flucytosine, CAS: caspofungin, AmB: amphotericin B. n = 3, error bars show standard deviation. Statistical significance was evaluated with Student's t-test. **P < 0.01.

***Manuscript in preparation*.** Please note that this manuscript hold preliminary data meaning that a few experiments await completion before the final manuscript is ready for submission.

Title

Cell state determines amphotericin B tolerance in yeast biofilm and planktonic populations

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Keywords

Yeast, fungi, biofilm, TOR, amphotericin B, rapamycin, tolerance, resistance, quiescence, starvation, antifungal, barcode sequencing, Bar-seq, persistence, stationary state

Author contributions

Designed the experiments: RB, DG, BR, AF

Performed the experiments: RB

Analyzed the data: RB DG BR AF

Wrote the paper: RB DG BR AF

ABSTRACT

Microbial biofilm formation is associated with decreased treatment success and persistent infections. Yeast infections are an increasing clinical problem and the ergosterol targeting fungicide amphotericin B is one of few antifungals with anti-biofilm activity. We show that a sub-population of *Saccharomyces cerevisiae* biofilm cells survived amphotericin B treatment. Re-inoculation of the surviving cells produced a similar proportion of drug tolerant cells, suggesting non-inherited resistance. To identify the molecular and physiological factors that contribute to the underlying amphotericin B tolerance in yeast biofilms, we screened a pooled collection of gene deletion mutants grown as biofilm or planktonic cells. Using multiplexed barcode sequencing (Bar-seq), we found that cells with mutations in genes involved in proliferation and metabolism were more tolerant to amphotericin B. The overlap between the specific genes selected for by growth in biofilm or planktonic growth was significant, and network analysis revealed overlap in the molecular pathways selected by specific modes of growth, suggesting that tolerance mechanisms are shared between biofilm and planktonic cells. We found that the level of tolerance to amphotericin B could be enhanced by prolonged starvation and rapamycin treatment. Our study contributes to the knowledge of the molecular mechanisms underlying biofilm drug tolerance and might lead to development of novel treatment strategies targeting quiescent cells.

INTRODUCTION

Advances in medical procedures have increased the use of invasive devices and immunosuppressive treatment, but at the same time have led to an increase in the number of patients susceptible to fungal infections [1]. It is believed that microbial biofilm formation is an important process in fungal infections [2]. The biomaterial of medical implants serves as compatible surfaces for attachment of fungal cells and the use of invasive devices are a risk factor for fungal biofilm infections [3]. The ability of biofilm cells to survive treatment with antimicrobial agents is a major challenge in treatment of infections and the molecular mechanisms that underlie drug tolerance of biofilm cells are not fully understood [4].

The majority of microbial cells in nature are thought to live in nutrient limited environments. Starvation for essential nutrients results in decreased metabolism and reduced cell growth. Decreased growth rate is correlated with increased stress tolerance [5,6] and stationary phase cells can survive exposure to a wide range of stress conditions [7]. The stationary state is defined by no net increase in population density [8] and includes heterogeneous population with phenotypic diverse sub-populations [9]. Even though the stationary state might be the most common growth state in nature, the knowledge about this phase is limited compared to our knowledge on exponential growth. Cells within biofilms have many phenotypic similarities with stationary state planktonic cells including low metabolism, phenotypic heterogeneity, and increased stress tolerance [10,11]. Few antifungal drug classes are available for treatment of systemic mycoses. The azoles account for the majority of total antifungal consumption and consequently an increase in non-susceptible *Candida* isolates have been reported [12]. Alternatives to the azoles are limited and only echinocandins and polyenes have *in vitro* activity against fungal biofilms [11,13,14].

The polyene drug amphotericin B has antifungal activity against a broad spectrum of fungal species making it the gold standard for treatment of severe mycoses. The drug targets ergosterol in the fungal cell membrane and form pores that result in rapid cell lysis [15]. The fungicidal properties of amphotericin B make it one of the few systemic antifungals with activity against growth arrested cells [11]. However, amphotericin B is unable to eradicate an entire population of biofilm and stationary state planktonic cells [11,16]. Because amphotericin B is the last in line treatment option, failure of amphotericin B treatment can have fatal consequences. Still, resistance to amphotericin B is rarely reported in the clinic and knowledge about the mechanisms is mainly limited to alterations of the target molecule or cell wall [15].

The genetically tractable *Saccharomyces cerevisiae* is closely related to the pathogenic yeast *Candida glabrata* [17] and is used as an experimental model for fungal biofilm studies [11,18,19]. A powerful molecular genetic tool available in *S. cerevisiae* is comprehensive barcoded gene-deletion strain collections which enable systematic studies of protein function and genotype to phenotype correlations [20]. The unique barcode tags of each mutant in combination with the advances of next generation sequencing create a

platform to perform cost efficient, high throughput, multiplexed screens of pooled mutants [21]. In this study, we used a gene deletion collection in a biofilm competent strain [22] to screen for mutants with increased tolerance to amphotericin B treatment when grown to starvation as planktonic or biofilm cells. We developed our experimental setup to be compatible with the suggested guidelines for design and analysis of Bar-seq experiments [23]. The biofilm and planktonic populations were treated with amphotericin B and samples were subsequently outgrown to enrich for viable cells. Genomic DNA was purified from the samples and the molecular barcodes that uniquely identifies each mutant were amplified and sequenced. Our results show that the level of drug tolerance could be induced and enhanced by prolonged starvation, exposure to rapamycin, or introduction of specific errors in metabolism and proliferation processes. A significant number of mutants were found to be tolerant in both biofilms and planktonic cultures. The gene products of the mutated genes uniquely identified in either mode of growth are involved in the same processes of the cell despite mode of growth, suggesting that identical mechanisms are responsible for drug survival in planktonic and biofilm populations.

MATERIALS AND METHODS

Strains

Saccharomyces cerevisiae Σ1278b YS-11 (*MAT***a** *can1Δ*::*STE2p-spHIS5 lyp1Δ*::*STE3p-LEU2 his3*::*HisG leu2Δ ura3Δ*) was used as reference wild type strain (Boone Lab, University of Toronto). The *S. cerevisiae* Σ1278b haploid gene-deletion collection (*MAT***a** *can1Δ*::*STE2p-spHIS5 lyp1Δ*::*STE3p-LEU2 his3*::*HisG leu2Δ ura3Δ*) [22] was transferred from 96-well glycerol stock plates to YPD agar and incubated at 30°C for three days. Colonies were subsequently pooled in YPD medium and incubated overnight in glass flasks with agitation at 30°C. Samples were spun down and dissolved in 5 ml YPD with 20% glycerol and frozen at -80°C.

Media and growth conditions

The Bar-seq experiment and all biofilm experiments were performed in minimal medium with 6.7 g/L Yeast Nitrogen Base without amino acids (BD Difco) and 2.0 g/L glucose supplemented with amino acids (Table S1). Yeast extract peptone dextrose (YPD) [24] medium was used for cultivation of planktonic cells except in the Bar-seq screen. All experiments were performed at 30°C.

Minimal inhibitory concentration

Minimal inhibitory concentrations (MIC) were determined as previously described [25] with modifications. In short, a two-fold dilution series of amphotericin B was prepared in fresh medium and distributed into 96-well microtiter plate. Visibly turbid overnight cultures were diluted to OD_{600} 0.1 in fresh medium and transferred to microtiter plates containing amphotericin B. Plates were incubated with agitation (60 rpm) at 30°C for 24 h and biomass was measured with a microplate spectrophotometer (BioTek PowerWave 340). Growth inhibition of $\geq$ 90% was determined as MIC as recommended by EUCAST [25]. MIC values were determined in YPD medium and minimal medium with 2% glucose.

Barcode sequencing

Cultivation

The pooled mutant collection was inoculated in quadruplicate to 700 ml minimal medium at density 5.0 ± 0.2 $\log_{10}$ CFU/ml. After 5 hours agitated incubation at 30°C, 50 ml samples were distributed to CELLSTAR Cell Culture Dishes (Cat. No. 639 160, Greiner Bio) and sealed with parafilm to avoid evaporation for biofilm growth, or glass flasks for planktonic growth. The cells were grown for 96 hours followed by 12 hours treatment with amphotericin B (see below).

Drug treatment

Amphotericin B (A2411, Sigma) was dissolved in DMSO in 5 mg/ml stock solution and kept at -20°C. After

96 hours cultivation of biofilm and planktonic cells, amphotericin B was added to a final concentration of 10 µg/ml, corresponding to ten times the MIC in minimal medium, directly to planktonic growing cells in the glass flasks. For biofilm growing cells, medium with non-attached cells was removed and centrifuged (10 minutes at 4500 G) and amphotericin B was added to the spent medium to a final concentration of 10 µg/ml before reintroduction to biofilm cells. Drug treated samples were shielded with alufoil to protect amphotericin B from light and incubated for 12 hours.

DNA extraction from viable mutants

Cells were harvested from biofilm growing cells by removing media with non-adhering cells, washing the biofilm-forming population in saline to remove excess planktonic and loosely adherent cells. Finally, biofilm cells were dissolved in saline and scraped off the surface with a Drigalski spatula and homogenized. Planktonic growing cells from glass flasks were centrifuged for 10 minutes at 4500 G and the cell pellets were washed once in saline. Cell pellets were dissolved in saline after an additional centrifugation step the. One ml of harvested cell culture was transferred to 25 ml YPD and outgrown at 30°C with agitation to enrich for viable mutants. Genomic DNA was extracted with the Wizard Genomic DNA Purification kit (A1125, Promega).

Library preparation

Amplification of molecular barcodes from genomic DNA and incorporation of the *Illumina P5* sequence was performed in a two-step PCR protocol as previously described with a few modifications [23]. In short, UPTAGs and DNTAGs were amplified in the first PCR reaction with unique index primers (Table S2) and purified PCR concentrations were estimated from gel band intensities. UPTAGs and DNTAGs were mixed in separate pools with 10 ng for each library. In a second PCR step, the *Illumina P5* sequence was incorporated to the UPTAGs and DNTAGs in two separate PCR reactions. The two PCR products were subsequently combined in equimolar amounts and diluted to a 2 nM final concentration. The library was sequenced on a single lane of an Illumina HiSeq 2000.

Data analysis

Sequence reads were matched to a library of known barcode sequences allowing single mismatch annotations [21]. The HO knockout strain was present multiple times in the 96-well stock plates that the deletion collection was pooled from. This strain accounted for 30% of total sequence reads and was consequently removed from the data set. UPTAG and DNTAG reads (Figure S1) within each biological replicate were summed and normalized to total reads per sample. Low abundant mutants were filtered if the mutant was present less than a 100 counts per million in less than four samples leaving a total of 2051 mutants for further data analysis. Because the variance of biological replicates was higher than the mean

(Figure S2), the data set followed an overdispersed Poisson distribution, and the edgeR package (version 3.2.4) was used for differential abundance analysis as previously described [23]. A false discovery rate (FDR) < 0.05 was considered significant.

Gene ontology

Genes found to be significantly overrepresented after amphotericin B treatment were manually assigned a biological function and grouped based on gene ontology as annotated by the *Saccharomyces* Genome Database (SGD). All gene ontology groups were verified by GO Term Finder from SGD.

Protein interaction network

Protein interaction network was made with the MiMI plugin (3.1) [26] to cytopscape (2.8.3) [27]. The network contain interactions between proteins from gene products of deleted genes that resulted in significant increased survival to amphotericin B. The network include proteins that interacts with at least one other protein found in the Bar-seq screen.

Phenotypic characterization of drug-tolerant biofilm sub-population

Yeast cultures with cell density OD_{600} 0.1 were distributed to the wells of a microtiter plate with a polystyrene surface. After two days static incubation, medium with planktonic and loosely adherent cells were removed and centrifuged (2 min at 10000 g). Biofilm cells were challenged for 24 hours with the indicated concentrations of amphotericin B added to the supernatant of the spent medium. Biofilm cells were washed twice in saline, re-dissolved and serial diluted in saline before plating on YPD agar. Inheritance of drug tolerant phenotype was investigated by re-dissolving amphotericin B (ten times MIC) exposed biofilm cells in fresh medium and transfers them to a new microtiter plate. After two days incubation, the biofilm population was exposed to amphotericin B (ten times MIC) in a total of three cycles identical to that just described.

Confocal microscopy

Visibly turbid cultures were diluted to OD_{600} 0.1 and transferred to biofilm chambers (Technical University of Denmark) with a polyvinyl chloride (PVC) coverslip surface (Rinzl, Electron Microscopy Sciences). After 96 hours static incubation at 30°C, medium was removed from biofilm chambers and centrifuged, and antifungal drug was added to the supernatants at 10 times the MIC. Spent medium with drug was introduced to the biofilm cultures followed by 12 hours incubation at 30°C. Rapamycin inhibition of biofilm cells was performed by incubation of fresh cultures with and without 1 µg/ml rapamycin for four hours followed by 3 hours amphotericin B treatment (10 times MIC). Staining for 15 minutes with 5 µM Syto9 (Invitrogen) was used to visualize live cells and 20 µM propidium iodide (Sigma-Aldrich) was used to stain dead cells.

Confocal laser scanning microscopy (CLSM) was performed with a Zeiss LSM710 microscope equipped with excitation lasers at 488 nm and 514 nm. Imaging used an EC Plan-Neofluar 40x/1.30 Oil lens.

Growth conditions for planktonic populations

Exponential growing yeast cells were dissolved in fresh pre-heated YPD medium to OD_{600} 0.1 in glass flasks. Half of the cultures were exposed to 1 µg/ml rapamycin (R0395, Sigma) from a 1000 µg/ml stock solution in DMSO, while the other half were kept untreated. The cultures were incubated at 30°C with agitation (200 rpm).

Growth curves

Samples were extracted for OD_{600} measurements before addition of rapamycin (t_0) and every hour post rapamycin treatment.

Drug susceptibility

Exponential growing rapamycin-untreated cells were exposed to five times the MIC amphotericin B, while the other half of the cells were pre-exposed to rapamycin for four hours before treatment with five times MIC of amphotericin B. CFUs were determined before and every hour post amphotericin B treatment for killing kinetics, and only after two hours in the mutant viability assay.

Starvation induced tolerance was investigated by diluting an exponential growing population to OD_{600} 0.01 in glass flasks and incubating it for 24 hours or five days with agitation, followed by treatment with 50 times MIC of amphotericin B for three hours. Viable cells were determined by CFUs.

RESULTS

Surviving cells are phenotypic variants of wild type

Amphotericin B has been reported to kill the majority of yeasts cells in a biofilm population, but reproducibly fails to eradicate a sub-population of cells that remain viable after treatment with 10 times the MIC [11].

To determine the correlation between drug concentration and cell density of the amphotericin B tolerant sub-population, biofilm cells were exposed to high concentrations of amphotericin B for 24 hours. Treatment with 10 μg/ml (10 times the MIC in minimal medium) resulted in survival of 5.0 ± 0.4 $\log_{10}$ CFU/ml (Figure 1A). Exposure to 50 μg/ml (50 times the MIC) decreased the population size to 4.9 ± 0.2 $\log_{10}$ CFU/ml and treatment with 100 μg/ml (100 times the MIC) resulted in 4.1 ± 0.4 viable $\log_{10}$ CFU/ml. No statistical significance was observed between treatment doses ($P > 0.05$, one-way ANOVA), showing that survival is not drug concentration-dependent.

We next investigated the inheritance of the amphotericin B tolerance phenotype. Yeast biofilms were exposed to 10 μg/ml of amphotericin B for 24 hours after which the surviving biofilm population was re-inoculated to form a new biofilm. The new biofilm was treated again and this procedure was repeated in a total of three inoculation and treatment cycles (Figure 1B). If the survival mechanism was mediated by resistant mutations, a significant increase in surviving cells would have been expected to occur after each treatment cycle. After the first cycle, 4.9 ± 0.2 $\log_{10}$ CFU/ml survived the amphotericin B treatment. The biofilm population produced by the surviving cells showed similar tolerance to amphotericin B as its progenitor with 5.3 ± 0.3 $\log_{10}$ CFU/ml. An additional cycle repeating this procedure showed that 4.5 ± 1.9 $\log_{10}$ CFU/ml survived exposure to amphotericin B. There was no significant difference between the size of the amphotericin B tolerant population ($P = 0.7314$, one-way ANOVA), suggesting that tolerance was not heritable.

Screening for Amphotericin B tolerant mutants using multiplexed barcode sequencing

Multiplexed barcode sequencing [23] was used to identify genes and processes that contribute to the generation of non-heritable tolerance of amphotericin B in nutrient starved planktonic and biofilm populations. The barcoded mutant collection was pooled and four biological replicates were inoculated to individual glass flasks followed by distribution to plastic surfaces for biofilm growth and to new glass flasks for planktonic growth (Figure 2A). After four days incubation, four biological replicates were treated with amphotericin B and four were kept untreated for both biofilm and planktonic populations. After 12 hours, cell samples for each biological replicate, treated and untreated, were extracted and outgrown to enrich for DNA from the surviving mutants prior to molecular barcodes sequencing.

We used multidimensional scaling (MDS) to investigate the concordance between biological replicates. We found that all biological replicates for untreated control samples grouped together and that both untreated control samples for biofilm and planktonic growth were found in the same cluster (Figure 2B). This shows high similarity in abundance of mutants between biofilm and planktonically grown populations. For the amphotericin B treated samples (triangles), three out of four biological replicates grouped together for both biofilm and planktonic grown cells. This indicates diverse selection of tolerant mutants upon amphotericin B treatment.

Mutant abundance after amphotericin B treatment

The pooled mutant collection grown as biofilm for four days reached a cell density of 6.8 ± 0.05 $\log_{10}$ CFU/ml (Figure 3A). After treatment with amphotericin B for 12 hours, the viable population decreased to 4.1 ± 0.5 $\log_{10}$ CFU/ml. Planktonic growth of the pooled mutant library resulted in a cell density of 7.0 ± 0.05 $\log_{10}$ CFU/ml after four days incubation. Amphotericin B treatment for 12 hours resulted in survival of 5.3 ± 0.2 $\log_{10}$ CFU/ml. The tolerant population was more than 10 times larger after planktonic growth compared to biofilm growth, indicating higher tolerance in planktonic cells. The higher standard deviation for drug treated samples compared to untreated further support the observation from the MDS plot (Figure 2B) that amphotericin B treatment increase heterogeneity of biological replicates.

We found 248 mutants to be significantly (FDR < 0.05) more abundant in biofilm cells after drug treatment (Table S3) and 179 were found for planktonic cells (Table S4). We found that 184 mutants were uniquely identified for biofilm and 115 were unique for planktonic cells (Figure 3B). A total of 64 mutants were found to be shared between the two modes of growth (Table S5), which was found to be significant ($P < 0.0001$, two-tailed Fischer's exact test) showing that the overlap was larger than expected by chance. This result indicates that *S. cerevisiae* has a large group of genes that confer amphotericin B tolerance in both biofilm and planktonic cultures.

Nine mutants were found to be hyper-susceptible to amphotericin B when grown as biofilm and two mutants were hyper-susceptible when cultivated as planktonic cells (Table S6). Three of the amphotericin B hyper-susceptible mutants identified for biofilm growth have deletions in genes with unknown function (Tda11, YEL057C, and YML003W). Additionally, Sas2 is involved in histone acetylation, Sim1 in cell wall organization, Gde1 in Glycerophosphocholine catabolism, and Dug3 in glutathione catabolism. The hyper-susceptible mutants identified in planktonic cultivated cells have gene deletions in the plasma membrane transporter Yor1 and the biotin synthesis gene Bio5. Because these mutants are unrelated and are not involved in overlapping biological processes, we chose to focus on mutants that increase survival in the rest of this study.

Deletion of protein synthesis and cell division cycle genes increase amphotericin B tolerance

We found 6% in biofilm and 5% in planktonic of the genes resulting in increased amphotericin B survival to be involved in lipid metabolism such as the ergosterol biosynthesis genes Ncp1, Cyb5 and Erg5 (Table 1, Table S7 and Table S8). The deletion of these genes likely results in structural modification of lipids leading to lowered affinity of ergosterol to amphotericin B [28]. Structural modification of ergosterol is a well described resistance mechanism against amphotericin B [15] and the identification of such mutants serves as positive validation of the Bar-seq screen.

The majority of identified mutants are affected in metabolic processes of the cell. Among them we found a large group of mutants with knockouts in genes involved in transcription and translation processes of the cell, including regulators of RNA polymerase II and ribosomal proteins (8 for biofilm cells and 11 for planktonic cells). Additionally, mutants deleted in metabolism of carbohydrates and small molecules such as carboxylic acids and amino acids showed increased amphotericin B tolerance.

We also observed a group of mutants involved in transport processes in the cell to increase amphotericin B survival. One-third of this group was involved in protein transport (34% in biofilm and 37% in planktonic), one-fourth in ion transport (24% in biofilm and 30% in planktonic), and carboxylic transport accounted for 15% in biofilm and 17% in planktonic cells.

Finally, we found that mutations in cell cycle processes increased amphotericin B tolerance. From this gene ontology, 41% for biofilm and 40% for planktonic were involved in cell cycle phase transition, while one-fifth was involved in G2/M transition of the mitotic cell cycle (18% for biofilm and 20% for planktonic), and one-fifth was involved in cytokinesis (23% for biofilm and 20% for planktonic).

These observations show that cells with increased tolerance may be impaired in central metabolic and cell proliferation processes.

Mutants have increased initial proportion of amphotericin B tolerant cells

To confirm the involvement of individual genes on drug tolerance we investigated the susceptibility of individually cultured mutants to amphotericin B treatment. We chose to focus on the shared genes between biofilm and planktonic cultivated cells, because this group was found to be significant and holds great therapeutic potential. We included *ssn3* (nucleic acid metabolism), *gin4* (cell cycle process), *slm1* (signal transduction), *erg5* (lipid metabolic process) and *sac1* (lipid metabolic process).

MIC determinations showed that only *erg5* had a reduction in amphotericin B susceptibility, while the remaining tested mutants all had MIC values similar to the wild type (Figure 4A). Because the MIC assay measures the ability to grow in the presence of an antifungal agent, these results suggested that the high level of amphotericin B tolerance of the mutants could be attributed to a non-growing cell state.

To investigate the initial population size of amphotericin B tolerant cells in the mutants, we treated exponential growing cells for two hours with amphotericin B and determined CFUs before and after

amphotericin B treatment. Treatment of exponential growing wild type cells with amphotericin B resulted in survival of 0.5% of the population (Figure 4B). Treatment of *slm1* resulted in survival of 0.3%, which was found to be significantly lower compared to wild type ($P < 0.05$, Student's t-test). Treatment of the mutants *gin4, erg5, ssn3,* and *sac1* all resulted in a significant increase in surviving populations ($P < 0.05$, Student's t-test). These results show that for *gin4, erg5, ssn3,* and *sac1*, significantly more cells are in a tolerant state during exponential growth compared to the wild type.

Inhibition of TORC1 increases amphotericin B tolerant population

The TOR pathway is responsible for regulation of growth and controlling metabolic processes in yeast cells in response to nutrient availability [29]. Inhibition of this pathway leads to growth arrest of cells in a quiescent G_0 state that can be induced by nutrient starvation or treatment with rapamycin [30]. Although *tor1* and *tor2* were not part of the screen, we found that deletion of members of the EGO complex (*gtr1, gtr2, meh1,* and *slm4*) that is required for exit of TOR-inhibited growth arrest [31] increased amphotericin B survival in our Bar-seq screen. Moreover, 20% of the shared genes between biofilm and planktonic cultivated cells that resulted in increased amphotericin B tolerance when deleted have previously been shown to have changed susceptibility to rapamycin, 81% of them being hyper-susceptible to rapamycin (Table S9) [32]. This indicate that the TOR inhibited response is induced with higher sensitivity in these mutants and shows that biofilm and planktonic cells share this pathway in tolerance to amphotericin B.

Treatment of the wild type with 1 µg/ml rapamycin resulted in growth arrest of exponential growing cells (Figure 5A). This allowed us to compare amphotericin B tolerance of exponential growing cells and TORC1-inhibited growth arrested cells. Rapid killing kinetics was observed after amphotericin B treatment of exponential growing cells resulting in killing of > 99% of the cells after three hours. Amphotericin B treatment of rapamycin-induced growth arrest resulted in slow killing with a ~500-fold increased amphotericin B tolerant population compared to exponential growing cells (Figure 5B). Rapamycin treatment was also found to be effective against growing yeast biofilm cells. Yeast cells were cultivated as biofilm for four hours with and without rapamycin. Rapamycin treated cells were growth inhibited and had a lower population density compared to untreated cells. Amphotericin B treatment of rapamycin exposed cells increased the ratio of surviving cells compared to untreated cells, showing that TORC1 inhibition result in increased survival of biofilm cells to amphotericin B treatment (Figure 5C).

Treatment with rapamycin inhibited the growth of yeast cells, but this might not fully represent the nature of nutrient starved cells. To investigate the effect on amphotericin B tolerance of naturally starved cells, a population of yeast cells cultured for one day (post diauxic shift) and five days (stationary phase) were exposed to 100 µg/ml amphotericin B for three hours (Figure 5D). Our results showed that prolonged starvation significantly ($P < 0.005$, Student's t-test) increased the amphotericin B tolerant sub-population.

In Figure 4B we observed that *slm1* did not contain a larger proportion of amphotericin B tolerant cells compared to wild type in exponential growth phase. However, incubation for 96 hours in minimal medium before amphotericin B treatment resulted in a significant higher level of amphotericin B tolerance in *slm1* biofilm compared to wild type biofilm cells ($P < 0.05$, Student's t-test) (Figure 6B). Similarly, when exponential growing planktonic cells were pre-exposed for four hours to rapamycin, significantly more *slm1* cells were amphotericin B tolerant compared to wild type ($P < 0.0001$, Student's t-test) (Figure 6B). These results indicate that nutrient starvation and TORC1 contribute to amphotericin B tolerance in *slm1*, while other additive factors are also responsible for tolerance in *gin4, erg5, ssn3,* and *sac1* (Figure S4).

Similar interaction networks lead to amphotericin B tolerance in biofilm and planktonic cultures

We created a protein interaction network of the gene products that when deleted uniquely resulted in amphotericin B tolerance unique for biofilm or planktonic growth to identify the molecular networks affecting growth specific amphotericin B tolerance. Of the 184 genes identified to increase tolerance in biofilms, 79 genes encode proteins that interact with at least one other protein found to be unique for either biofilm or planktonic growth (Figure 7). Only four of them were found to not have at least a third degree interaction with a protein identified to be unique for planktonic cells. For proteins identified in the planktonic screen, 52 proteins were found to interact with at least one other protein identified to be growth specific in the Bar-seq screen. Only two proteins were found to not have at least a third degree interaction with a protein found to be unique for biofilm cells. We found a substantial overlap in molecular networks affecting amphotericin B tolerance under both growth conditions. Although the majority of mutants were found to uniquely increase survival during either biofilm or planktonic mode of growth, the gene products of the knockouts interact with each other independent of mode of growth, suggesting that they are involved in the same pathways. Moreover, genes identified in biofilm or planktonic growth could be assigned to the same biological function gene ontologies (Table 1). We found ~1.5-fold more genes that upon knockout resulted in increased tolerance to amphotericin B when grown as biofilm compared to planktonic cultivation. This fold difference recurs in almost all biological function groups and each group account for similar percentages of the total significant genes (Table 1). These observations strongly imply that even though more gene deletions were found to uniquely increase amphotericin B survival in biofilm cells compared to planktonic cells, the genes are involved in the same biological processes.

DISCUSSION

The molecular mechanisms underlying antimicrobial drug tolerance of biofilm cells are major puzzles in modern medicine. Attempts to identify biofilm specific mechanisms that can explain multidrug tolerance have been inconsistent [33]. Research in bacteria have shown that nutrient limitation in biofilm and planktonic cells activate a starvation response that increase antimicrobial tolerance showing that antimicrobial drug tolerance mechanisms are shared between nutrient starved biofilm and planktonic bacteria [34-36]. We have recently shown that planktonic and biofilm yeast cells share similar tolerance phenotypes when grown to starvation [11]. In the present study, we investigated the molecular mechanisms responsible for tolerance to the fungicidal drug amphotericin B in nutrient starved population of biofilm and planktonic yeasts. Using multiplexed barcode sequencing [23] of a pooled mutant collection in a biofilm competent strain [22], we identified genes involved in cell metabolism and proliferation to be involved in tolerance to this drug regardless of mode of growth.

The Bar-seq screen identified genes involved in four major cellular processes. Not surprisingly were genes involved in lipid metabolism identified as affecting amphotericin B tolerance such as the ergosterol synthesis genes *ERG5*, *NCP1* and *CYB5*. Deletion of these genes is likely to result in decreased affinity of the drug to the plasma membrane. In addition to lipid metabolism, the screen identified that deletion of transcriptional and translational genes increased survival to amphotericin B treatment. Moreover, genes involved in vesicle mediated transport of proteins and membrane components between the intracellular compartments affected amphotericin B tolerance and suggest a role for intracellular remodeling or cytoplasmic membrane maintenance in survival. Furthermore, mutations in genes involved in the mitotic cell cycle indicate that the tolerant sub-populations could have arrested growth at different cell cycle phases.

We found a total of 363 gene deletions to increase survival for yeast cells grown as biofilms or planktonically. The large diversity of cellular processes that can affect amphotericin B tolerance is similar to the wide range of mechanism that can generate antimicrobial persisters in bacteria, which has been hypothesized to be the result of fluctuating variability in cellular processes [37]. The many different kinds of errors that can cause phenotypic variability and the different ways to induce persistence suggests that persistence is not a unique state, but rather a collection of physiological distinct cellular processes that each are associated with a drug tolerance phenotype [37,38]. Our results indicate that stochastic variations in cell metabolism and cell cycle processes could be the cause of phenotypic heterogeneous populations with amphotericin B tolerant subpopulations. We observed that some of the mutants had a higher initial proportion of amphotericin B tolerant cells compared to wild type, and the MIC values indicate that this subpopulation could be growth arrested.

Ceased proliferation and increased stress survival in yeast cells can be attributed to a quiescent state of cell cycle arrest [7]. Quiescence can be induced by exposure to stress, and is well characterized for stationary

state populations. Stationary phase populations contain both quiescent and non-quiescent cells that can be distinguished by the ability of quiescent cells to survive for extended periods of starvation and resume proliferation when the growth conditions become favorable [9,39]. Similar to *Candida albicans* biofilm [16], we found that a subpopulation of the starved cells survived amphotericin B treatment and that it was phenotypic variants of the wild type, rather than resistant mutants that survived (Figure 1). We furthermore observed that the amphotericin B tolerant subpopulation resumed growth when reinoculated to fresh medium without drug, showing that viable cells have quiescent cell properties. Recent investigations indicate that yeast cells can access distinct quiescent states [40] that can be arrested at all cell cycle phases [41]. Therefore it seems that quiescence is not a pre-programmed state, but rather a result of slow metabolism that result in growth arrest [40,41]. Production of heterogeneous populations of quiescent cells might result in subpopulations with different levels of tolerance to amphotericin B caused by specific defects that we observed in this study (Figure 4B). It is therefore possible that the genes we identified in the Bar-seq screen are not directly involved in tolerance but rather affect the proportion of cells entering and leaving the quiescent state.

We found that the transition from post diauxic shift to stationary phase significantly enhanced amphotericin B tolerance (Figure 5D). This result indicates that nutrient depletion associated with stationary phase entry resulted in acquired stress tolerance to amphotericin B. The highly conserved TOR signaling pathway controls cell growth in response to nutrient availability. Starvation for carbon or nitrogen inhibit TOR and result in quiescent cell cycle arrest [29]. Treatment with the immunosuppressive drug rapamycin inhibits TOR complex 1 (TORC1) and induces a quiescent transcriptional program in yeast [30]. We showed that pre-exposure to rapamycin increased survival to amphotericin B treatment by ~500-fold (Figure 5B), and that the entire population of *slm1* became tolerant to amphotericin B after pre-exposure to rapamycin (Figure 6C). These results show a role of the TOR signaling pathway in amphotericin B tolerance and that this is a shared mechanism between planktonic and biofilm starved populations. Furthermore, exit of TOR inhibited quiescence involves members of the EGO protein complex [31]. We found the EGO mutants *gtr1*, *gtr2*, *meh1*, and *slm4* to increase survival of amphotericin B treatment in glucose starved planktonic and biofilm cells. The mutants identified to increase amphotericin B tolerance in the Bar-seq screen not only suggest that phenotype diversity that facilitates higher susceptibility to enter a quiescent state, but also to exit this phase again, are key tolerance mechanisms.

We suggest that amphotericin B tolerance can be achieved by stochasticity in cell metabolism and cell proliferation processes and that the proportion of tolerant cells can be enhanced by starvation conditions in a TOR-dependent manner. We identified *ras2* in both biofilm and planktonic cells and *ras1* grown as biofilm to increase amphotericin B survival in the Bar-seq screen. It is therefore likely that other nutrient sensing systems like the Ras/PKA pathway are also involved in generating amphotericin B tolerance.

We have found that only a sub-population of growth arrested cells are tolerant to amphotericin B and that this sub-population contain cells with different kinds of defects and different levels of tolerance. What distinguish tolerant cells from susceptible cells is likely to be alterations specific for the mechanism of action of amphotericin B. Amphotericin B target membrane ergosterol and form pores that cause rapid cell lysis. The yeast cell membrane and cell wall are altered in response to starvation to protect the cell from environmental stresses and ensure survival during prolonged starvation [7]. Since the overall metabolism of the cell is decreased during quiescence, catabolism of energy storage is required to remain viable. Fatty acid oxidation is increased in quiescent cells probably for the utilization of fatty acids as energy source during starvation [9,39,42]. Since fatty acids are precursors for membrane lipids, it is likely that quiescent cells have physiochemical changed membranes with decreased affinity to amphotericin B either by lipid raft alterations or structural modifications of ergosterol. From our screen we did indeed observe mutations in lipid metabolism to be an important factor in amphotericin B tolerance during starvation.

One important conclusion of this study is that biofilm and planktonic populations have similar amphotericin B tolerance mechanisms when pre-exposed to the same stress such as nutrient starvation or rapamycin treatment. This has also been observed in bacteria [34-36] and strongly suggest that the drug tolerance associated with biofilms is attributed to the nutrient starved conditions they are exposed to in the lab (i.e. glucose limited medium) [18,43] or in the human host [36]. Antimicrobial tolerance of biofilm cells is a consequence of environmental conditions rather than biofilm-specific adaptation. We have shown in this study that planktonic and biofilm yeast cells pre-exposed to identical environmental stresses respond similarly to antifungal treatment.

Our study contribute to the current knowledge about antifungal tolerance of biofilm cells and further insight into quiescent mediated drug tolerance might lead to development of novel treatment strategies that target quiescent cells. Understanding survival of quiescent cells is not limited to pathogenic microbes as it is also relevant in treatment of dormant cancer cells [44].

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576

577 **TABLES**

578

579 **Table 1.** GO annotations from tolerant mutants identified in the Bar-seq screen.
580

GO	Biological function	Biofilm (%)	Planktonic (%)
6810	Transport	42 (17)	30 (17)
90304	Nucleic acid metabolic process	28 (11)	33 (18)
22402	Cell cycle process	22 (9)	10 (6)
19538	Protein metabolic process	18 (7)	10 (6)
6629	Lipid metabolic process	15 (6)	9 (5)
6412	Translation	13 (5)	11 (6)
44281	Small molecule metabolic process	13 (5)	8 (4)
7165	Signal transduction	8 (3)	4 (2)
5975	Carbohydrate metabolic process	6 (2)	6 (3)
50896	Response to stimulus	5 (2)	6 (3)
71555	Cell wall organization	2 (1)	7 (4)
45333	Cellular respiration	3 (1)	3 (2)
N/A	Other	13 (5)	9 (5)
N/A	Unknown	51 (21)	31 (17)

581

FIGURES

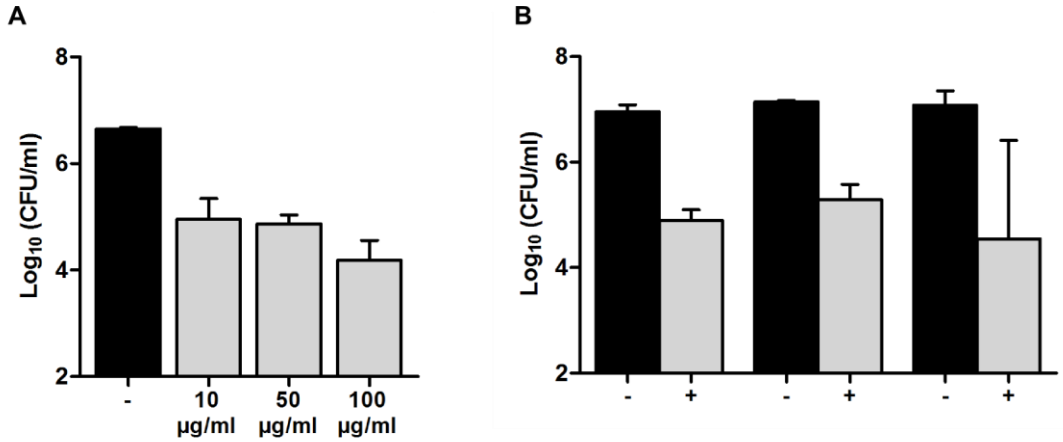


Figure 1. Cells that survive amphotericin B treatment are phenotypic variants of wild type . Cultures were grown for 48 hours as biofilms and viability was measured as colony-forming units (CFUs). Cells were treated with amphotericin B, or left untreated as a control (-), and CFUs were measured after 24 hours. (**A**) Viability of *S. cerevisiae* wild type cells after treatment with increasing amphotericin B concentrations. (**B**) Survival of *S. cerevisiae* wild type cells after treatment with 10 µg/ml amphotericin B (+). The surviving population was reinoculated to form a new biofilm that again was exposed to amphotericin B. This was performed in a total of three inoculation and treatment cycles. n = 3, error bars show standard deviation.

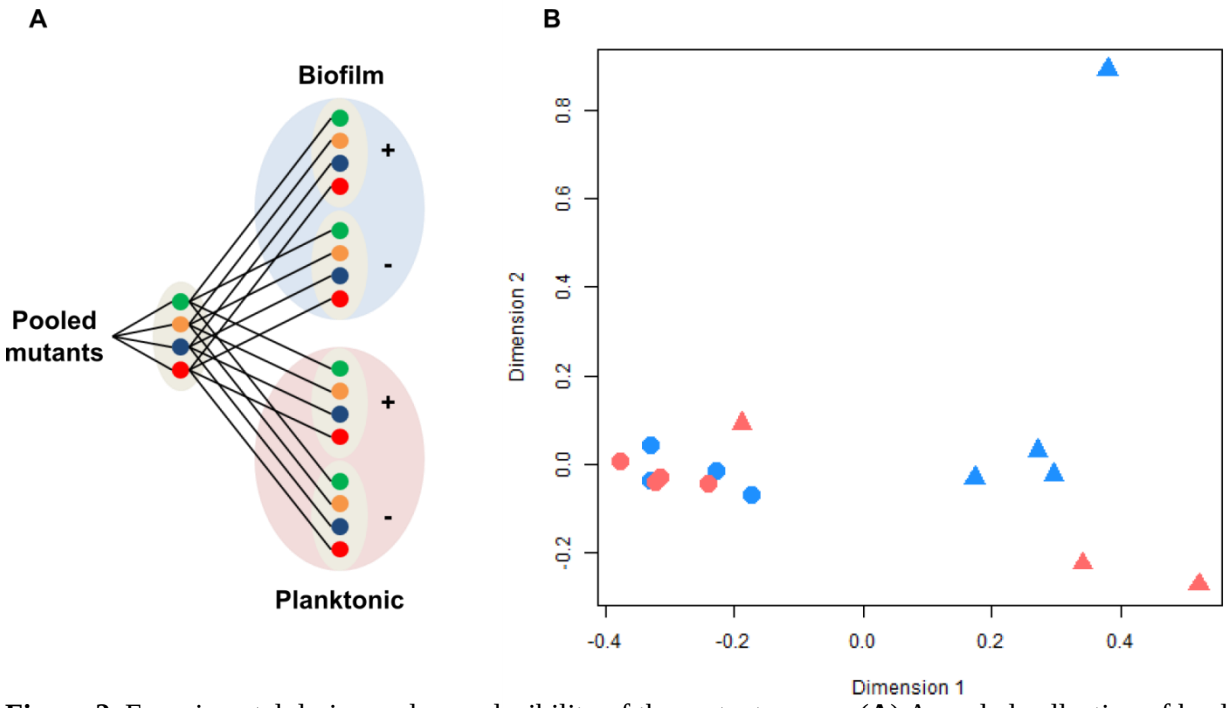


Figure 2. Experimental design and reproducibility of the mutant screen. (**A**) A pooled collection of haploid gene deletion mutants was cultivated as biofilm or planktonic cells in four biological replicates (colored circles). After four days, half of the biofilm or planktonic populations were treated with 10 µg/ml amphotericin B for 12 hours (+) or left untreated as control (-). Surviving cells were enriched by outgrowth in YPD medium before barcode sequencing. (**B**) Multidimensional scaling plot show the reproducibility of the four biological replicates. Untreated biofilm cells (blue circles), untreated planktonic cells (red circles), amphotericin B treated biofilm cells (blue triangles), and amphotericin B treated planktonic cells (red triangles).

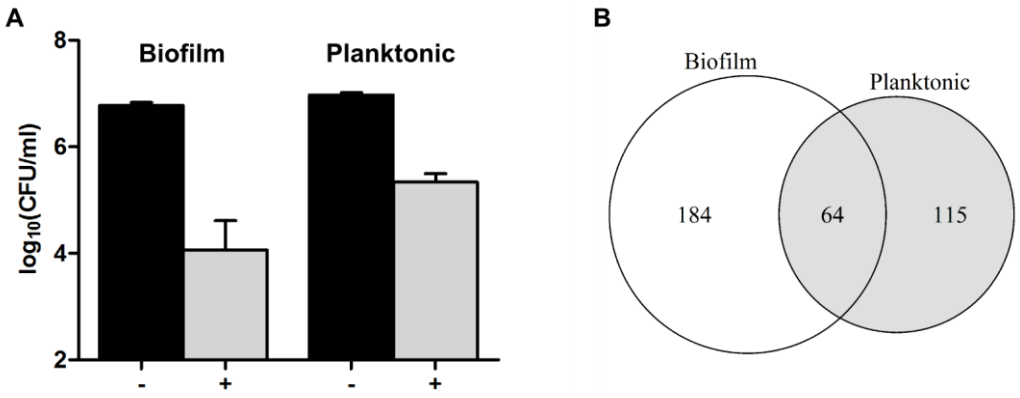


Figure 3. Mutant abundance after amphotericin B treatment. The pooled mutant collection cultivated as biofilm or planktonic cells for four days and treated with amphotericin B for 12 hours. (**A**) Number of viable cells measured as CFUs of untreated control (-) and amphotericin B treated cells (+). n = 4, error bars show standard deviation. (**B**) Venn diagram showing the overlap of mutants found to be tolerant to amphotericin B treatment after barcode sequencing. The overlap was significant ($P < 0.0001$, two-tailed Fischer's exact test).

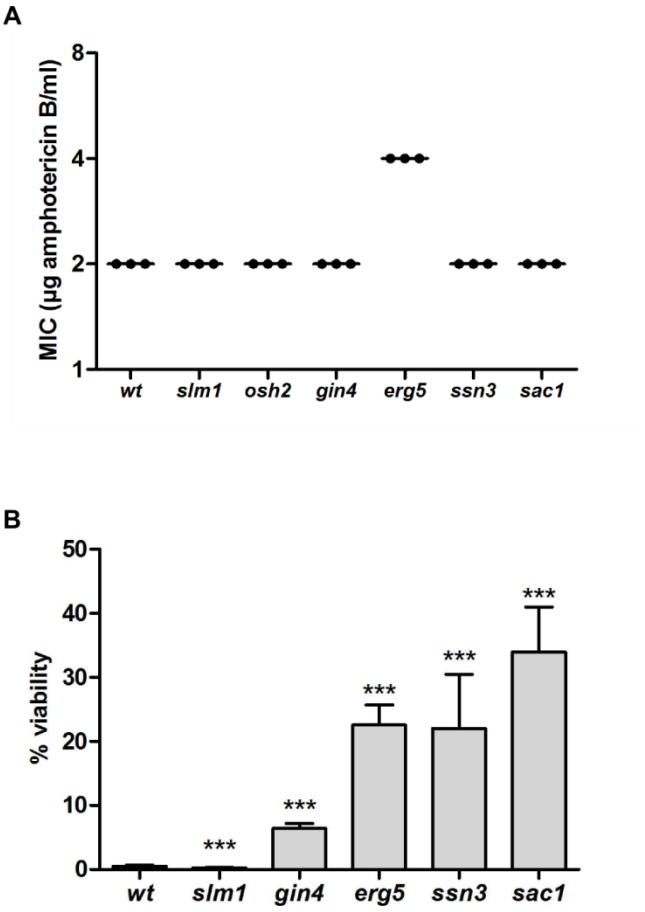
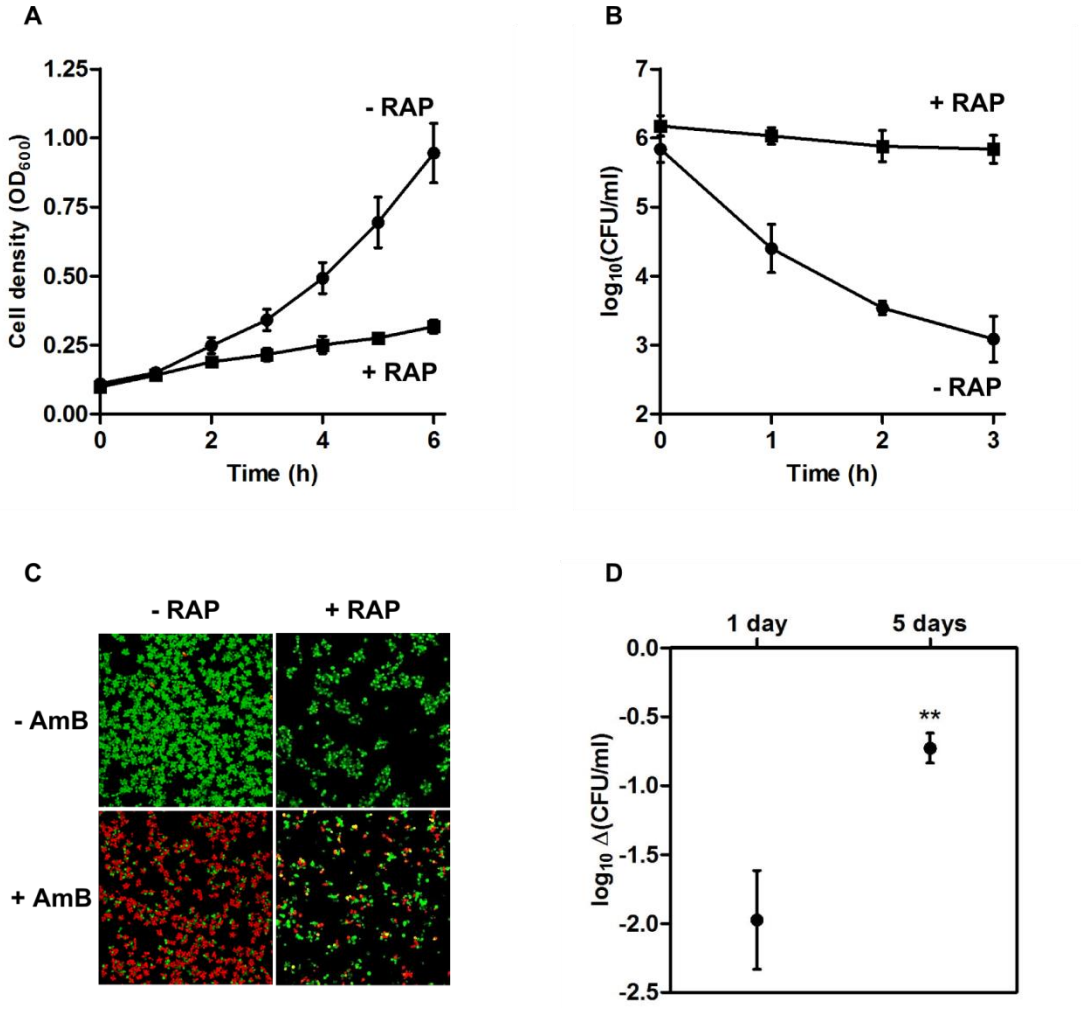


Figure 4. Amphotericin B tolerant deletion mutants are more susceptible to enter a quiescent state compared to wild type. (**A**) Minimal inhibitory concentrations of amphotericin B to selected mutants. Each point represents an individual experiment. (**B**) Survival of exponential growing wild type and mutants to two hours amphotericin B treatment (10 µg/ml). Survival was determined by the percentage difference in CFUs of amphotericin B treated and untreated cells. n = 3, error bars show standard deviation. Statistical significance was evaluated with Student's t-test. ***P < 0.05. Survival was determined by the percentage difference in CFUs of amphotericin B treated and untreated cells. n = 3, error bars show standard deviation.



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635 **Figure 5.** TOR inhibition mediates amphotericin B tolerance. (**A**) Planktonic cells grown in YPD were
636 treated with 1 µg/ml rapamycin (+ RAP) or left untreated (- RAP). Samples were extracted every hour and
637 OD_{600} was measured for cell density. n = 3, error bars show standard deviation (**B**) Exponential growing
638 planktonic cells in YPD were treated with 1 µg/ml rapamycin (+ RAP) for four hours before exposure to 10
639 µg/ml amphotericin B or only treated with 10 µg/ml amphotericin B (- RAP). CFUs were determined every
640 hour. n = 3, error bars show standard deviation. (**C**) Amphotericin B activity against four hour biofilm after
641 pre-exposure to 1 µg/ml rapamycin (+ RAP) or no rapamycin pre-exposure (- RAP). (**D**) Survival of 100
642 µg/ml amphotericin B treatment for three hours of planktonic cells incubated for one or five days. n = 3,
643 error bars show standard deviation. Statistical significance was evaluated with Student's t-test. **P < 0.005.

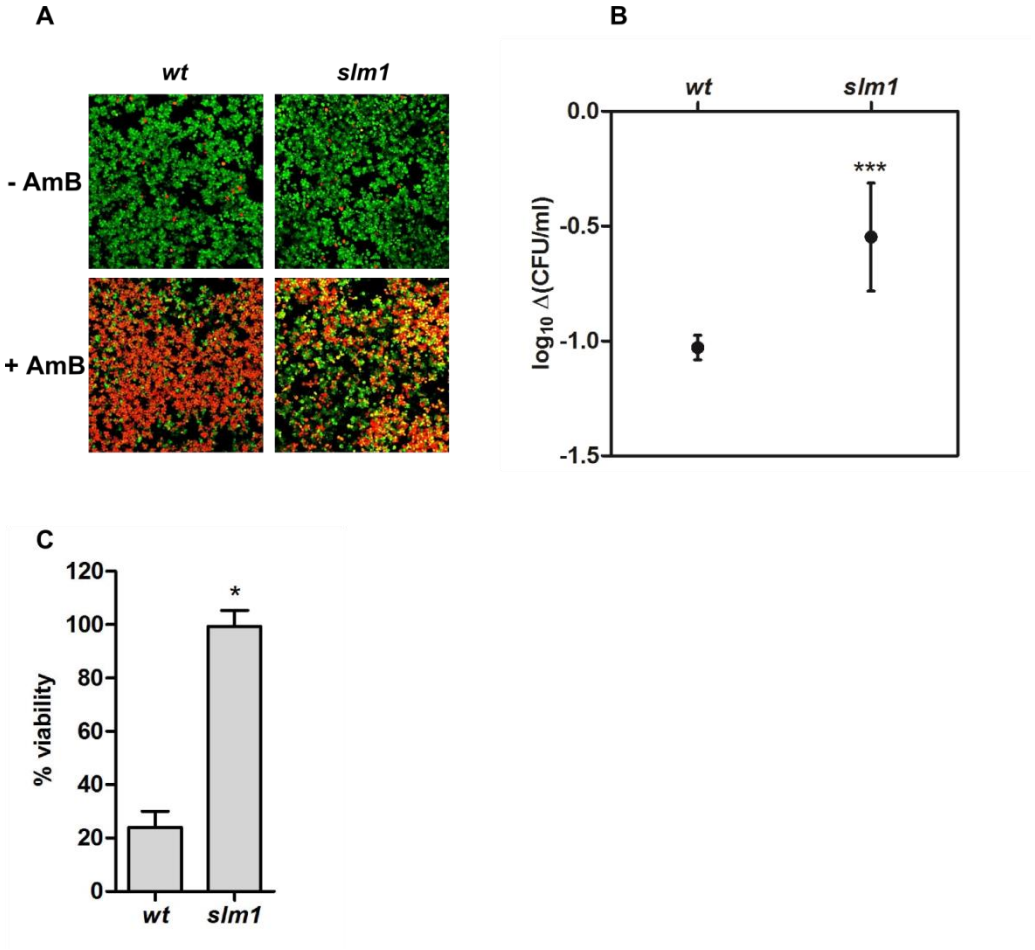

Figure 6. Starvation and rapamycin enhance amphotericin B survival. Wild type and *slm1* were cultivated individually as biofilms for four days followed by 12 hours treatment with amphotericin B or left untreated. (**A**) Visualization of surviving (green) and dead (red/yellow) cells after LIVE/DEAD staining and confocal laser scanning microscopy. (**B**) Quantification of viable cells after amphotericin B treatment. Shown is log change in viability between treated and untreated cells. n = 3, error bars show standard deviation. Statistical significance was evaluated with Student's t-test. ***P < 0.05. (**C**) Planktonic cells were pre-exposed to 1 µg/ml rapamycin for four hours before two hours amphotericin B treatment (10 µg/ml). Survival was determined by the percentage difference in CFUs of amphotericin B treated and untreated cells. n = 3, error bars show standard deviation. Statistical significance was evaluated with Student's t-test. *P < 0.0001.

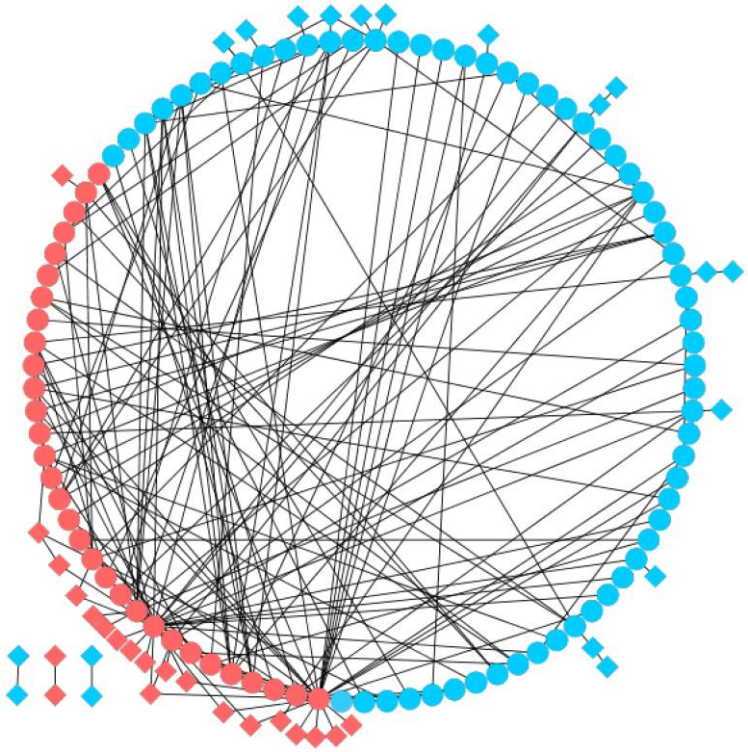

Figure 7. Similar interaction networks lead to amphotericin B tolerance in biofilm and planktonic cultures. Protein interaction network of gene products from deleted genes found to uniquely confer tolerance to either biofilm (blue) or planktonic (red) cells. The network only includes proteins that interact with at least one other protein found in the Bar-seq screen. Circular symbols represents a direct interaction between two protein found to be unique for the opposite mode of growth. Diamond symbols in the periphery of the circular network represent protein with a second or third degree interaction with a protein found to be unique to the opposite mode of growth. Diamond symbols not attached to the circular network only interact with a protein found to be unique to the same mode of growth.

663 **SUPPORTING INFORMATION**

664

665 **Table S1.** Amino acid concentrations used in minimal medium

Supplement	Medium concentration (mg/l)	Supplement	Medium concentration (mg/l)
L-Adenine	35	L-Leucine	180
L-Alanine	120	L-Lysine	180
L-Arginine	120	L-Methionine	75
L-Aspargine	120	L-Phenylalanine	75
L-Aspartic acid	120	L-Proline	120
L-Cysteine	180	L-Serine	120
L-Glutamic acid	180	L-Threonine	150
L-Glutamine	120	L-Tryptophan	120
Glycine	120	L-Tyrosine	45
L-Histidine	120	L-Valine	180
L-Isoleucine	180	Uracil	120

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667

668 **Table S2.** Primers used for barcode sequencing [23]

Primer Name	Primer Sequence
Illumina UPTAG Index 1	ACG CTC TTC CGA TCT ATACC GTC CAC GAG GTC TCT
Illumina DNTAG Index 1	ACG CTC TTC CGA TCT ATACC GTG TCG GTC TCG TAG
Illumina UPTAG Index 19	ACG CTC TTC CGA TCT GGAAC GTC CAC GAG GTC TCT
Illumina DNTAG Index 19	ACG CTC TTC CGA TCT GGAAC GTG TCG GTC TCG TAG
Illumina UPTAG Index 20	ACG CTC TTC CGA TCT CTCAG GTC CAC GAG GTC TCT
Illumina DNTAG Index 20	ACG CTC TTC CGA TCT CTCAG GTG TCG GTC TCG TAG
Illumina UPTAG Index 21	ACG CTC TTC CGA TCT ACTGG GTC CAC GAG GTC TCT
Illumina DNTAG Index 21	ACG CTC TTC CGA TCT ACTGG GTG TCG GTC TCG TAG
Illumina UPTAG Index 22	ACG CTC TTC CGA TCT TACAT GTC CAC GAG GTC TCT
Illumina DNTAG Index 22	ACG CTC TTC CGA TCT TACAT GTG TCG GTC TCG TAG
Illumina UPTAG Index 23	ACG CTC TTC CGA TCT GGCAT GTC CAC GAG GTC TCT
Illumina DNTAG Index 23	ACG CTC TTC CGA TCT GGCAT GTG TCG GTC TCG TAG
Illumina UPTAG Index 24	ACG CTC TTC CGA TCT CAGTT GTC CAC GAG GTC TCT
Illumina DNTAG Index 24	ACG CTC TTC CGA TCT CAGTT GTG TCG GTC TCG TAG
Illumina UPTAG Index 25	ACG CTC TTC CGA TCT ATACC GTC CAC GAG GTC TCT
Illumina DNTAG Index 25	ACG CTC TTC CGA TCT ACCAG GTG TCG GTC TCG TAG
Illumina UPTAG Index 26	ACG CTC TTC CGA TCT TTAGC GTC CAC GAG GTC TCT
Illumina DNTAG Index 26	ACG CTC TTC CGA TCT TTAGC GTG TCG GTC TCG TAG
Illumina UPTAG Index 27	ACG CTC TTC CGA TCT GCCAC GTC CAC GAG GTC TCT
Illumina DNTAG Index 27	ACG CTC TTC CGA TCT GCCAC GTG TCG GTC TCG TAG
Illumina UPTAG Index 28	ACG CTC TTC CGA TCT CGACC GTC CAC GAG GTC TCT
Illumina DNTAG Index 28	ACG CTC TTC CGA TCT CGACC GTG TCG GTC TCG TAG
Illumina UPTAG Index 70	ACG CTC TTC CGA TCT TGGTG GTC CAC GAG GTC TCT
Illumina DNTAG Index 70	ACG CTC TTC CGA TCT TGGTG GTG TCG GTC TCG TAG
Illumina UPTAG Index 71	ACG CTC TTC CGA TCT GTCCT GTC CAC GAG GTC TCT
Illumina DNTAG Index 71	ACG CTC TTC CGA TCT GTCCT GTG TCG GTC TCG TAG
Illumina UPTAG Index 72	ACG CTC TTC CGA TCT CCTTA GTC CAC GAG GTC TCT
Illumina DNTAG Index 72	ACG CTC TTC CGA TCT CCTTA GTG TCG GTC TCG TAG
Illumina UPTAG Index 95	ACG CTC TTC CGA TCT GCATA GTC CAC GAG GTC TCT
Illumina DNTAG Index 95	ACG CTC TTC CGA TCT GCATA GTG TCG GTC TCG TAG
Illumina UPTAG Index 96	ACG CTC TTC CGA TCT CCGTC GTC CAC GAG GTC TCT
Illumina DNTAG Index 96	ACG CTC TTC CGA TCT CCGTC GTG TCG GTC TCG TAG
Illumina UPkanMX	CAA GCA GAA GAC GGC ATA CGA GAT GTC GAC CTG CAG CGT ACG
IlluminaDNkanMX	CAA GCA GAA GAC GGC ATA CGA GAT ACG AGC TCG AAT TCA TCG
Illumina P5	A ATG ATA CGG CGA CCA CCG AGA TCT ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT

669

670

Table S3. Fold change in mutant abundance before and after amphotericin B treatment when cells were grown as biofilms.
Available from: https://dl.dropboxusercontent.com/u/55799248/TableS3.xlsx

Table S4. Fold change in mutant abundance before and after amphotericin B treatment when cells were grown planktonically.
Available from: https://dl.dropboxusercontent.com/u/55799248/TableS4.xlsx

Table S5. List of mutants that confer amphotericin B tolerance in both biofilm and planktonic populations.
Available from: https://dl.dropboxusercontent.com/u/55799248/TableS5.xlsx

Table S6. Hyper-susceptible mutants to amphotericin B identified in the Bar-seq experiments

Growth	ORF/gene	n (%)
Biofilm	YHR159W/TDA11 YMR127C/SAS2 YEL057C YIL123W/SIM1 YNL191W/DUG3 YPL110C/GDE1 YML003W YDR440W/DOT1 YGL220W/FRA2	9 (4)
Planktonic	YGR281W/YOR1 YNR056C/BIO5	2 (1)

Table S7. GO term annotations of amphotericin B tolerant mutants grown as biofilms.
Available from: https://dl.dropboxusercontent.com/u/55799248/TableS7.xlsx

Table S8. GO term annotations of amphotericin B tolerant mutants grown planktonically.
Available from: https://dl.dropboxusercontent.com/u/55799248/TableS8.xlsx

690 **Table S9.** Rapamycin sensitivity. "-" denotes hyper-sensitivity to rapamycin, "+" denotes resistance to
691 rapamycin. Data from [32]. "x" denotes if a mutant with changed rapamycin sensitivity was observed to
692 result in amphotericin B tolerance.

ORF Name	Gene name	RapamycinS	Both	Biofilm	Planktonic
YNL111C	CYB5	-	x		
YDR069C	DOA4	-	x		
YGL043W	DST1	-	x		
YMR015C	ERG5	-	x		
YDR507C	GIN4	+	x		
YML121W	GTR1	-	x		
YGR163W	GTR2	-	x		
YBR077C	NIR1	-	x		
YJL168C	SET2	-	x		
YPL042C	SSN3	+	x		
YOL018C	TLG2	-	x		
YML001W	YPT7	-	x		
YKR007W	MEH1	-	x		
YPL150W	N/A	+	x		
YCR094W	CDC50	-		x	
YOR358W	HAP5	+		x	
YBR133C	HSL7	-		x	
YHR064C	PDR13	-		x	
YBR284W	N/A	-		x	
YNL297C	MON2	-			x
YMR029C	FAR8	-			x
YNR010W	CSE2	-			x
YGL023C	PIB2	-			x
YGR129W	SYF2	+			x
YHR034C	PIH1	-			x
YHR045W	N/A	-			x

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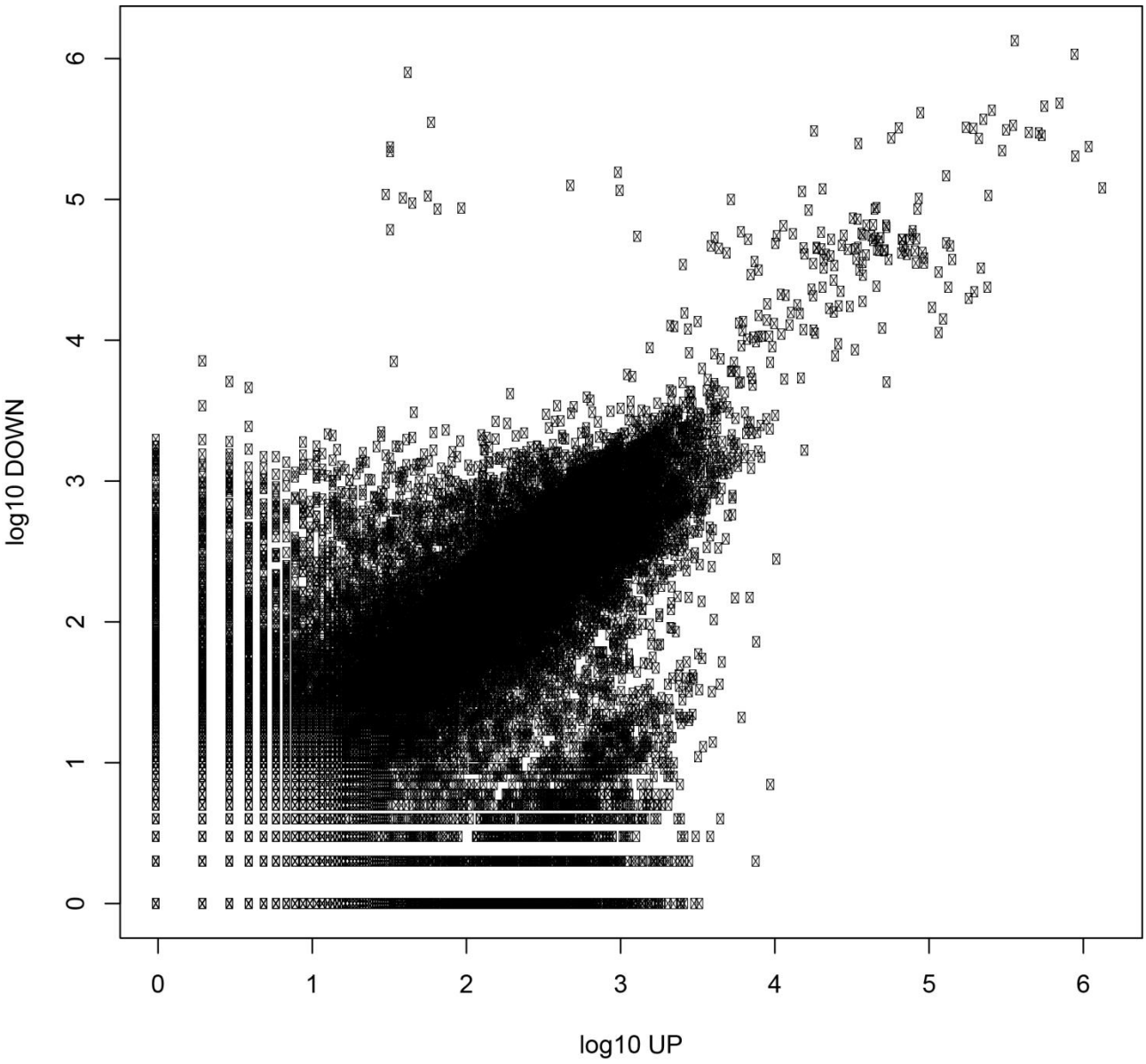

695

696 **Figure S1.** Sequencing reads of uptag and downtag barcodes

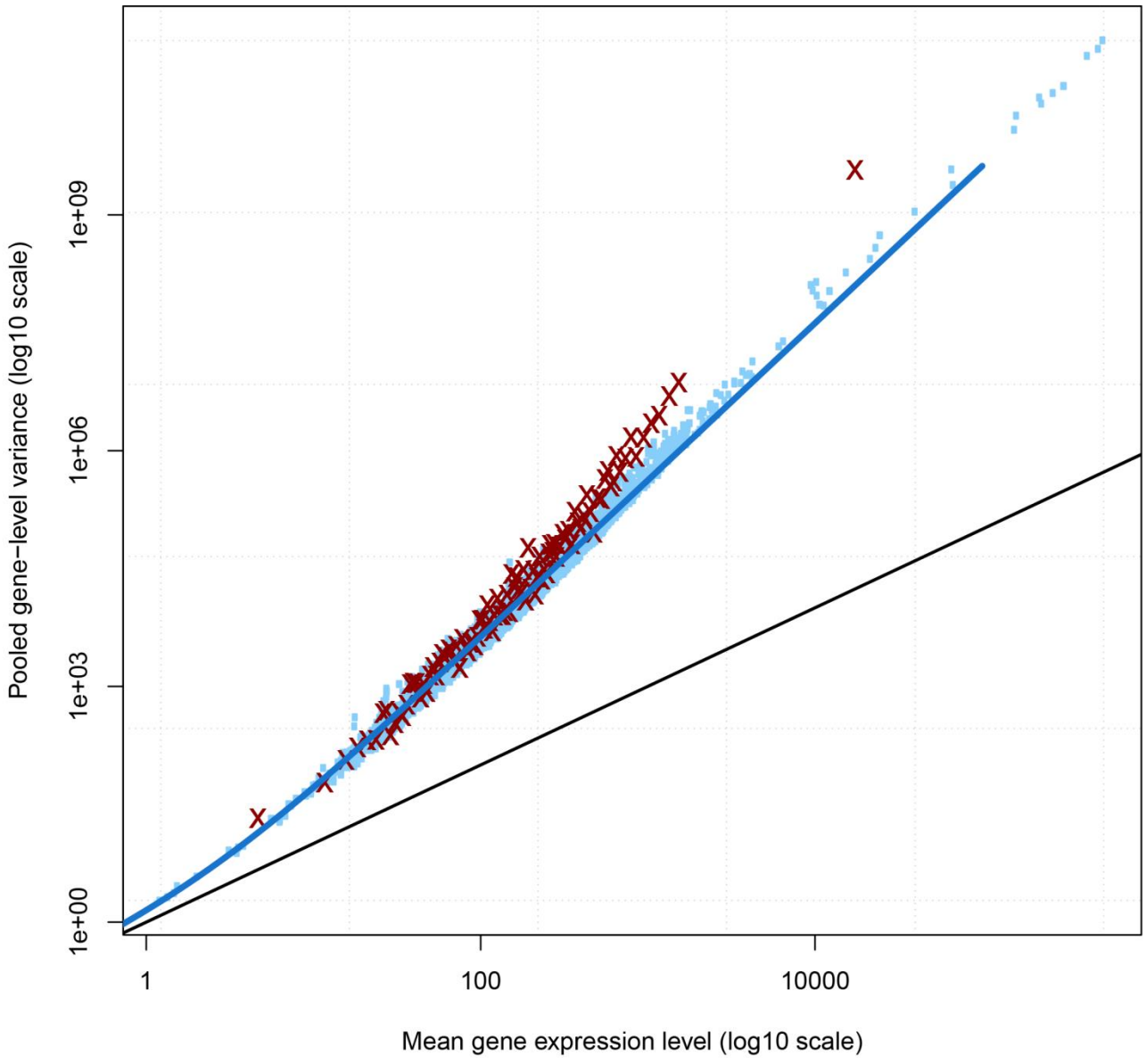

Figure S2. Comparison of mean barcode count with associated variance. Grey points are the raw measurements for each mutant, the red X's are the average variance in each bin, and the blue points are the estimated variance of each mutant after dispersion shrinkage has been performed.

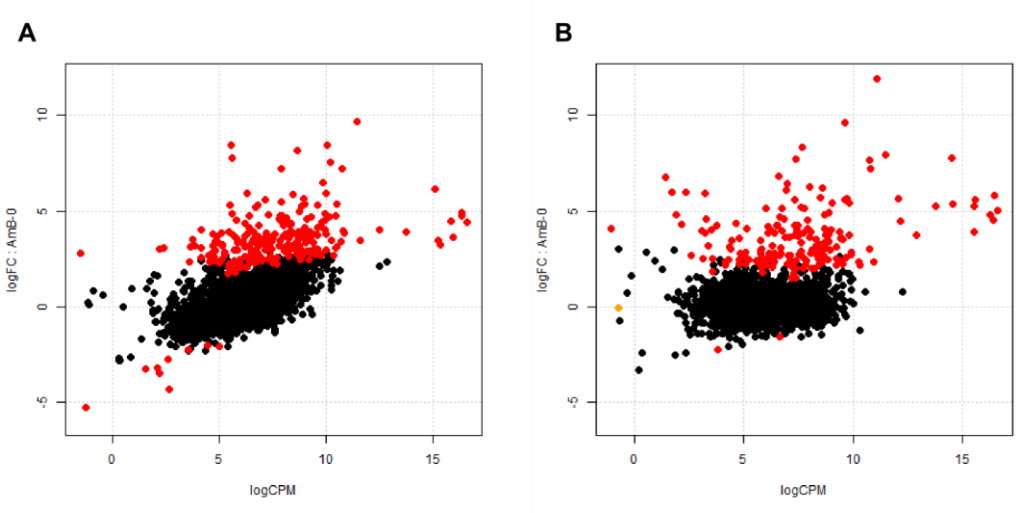

Figure S3. Log_{10} fold change (logFC) in sequencing reads between drug treated and untreated samples, and the total abundance of each mutant in the mixed population measured as log_{10} counts per million (logCPM). Smear plot showing the correlation between mutant abundancy after amphotericin B treatment and total number of sequencing counts for biofilm (**A**) and planktonic (**B**) grown cells.

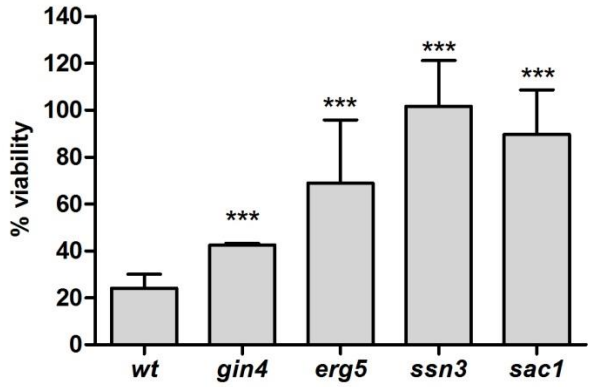

Figure S4. Inhibition of TOR is and additive effect in amphotericin B survival. Planktonic cells were pre-exposed to 1 µg/ml rapamycin for four hours before two hours amphotericin B treatment (10 µg/ml). Survival was determined by the percentage difference in CFUs of amphotericin B treated and untreated cells. n = 3, error bars show standard deviation. Statistical significance was evaluated with Student's t-test. ***P < 0.05.

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PLOS ONE

The Synthetic Amphipathic Peptidomimetic LTX109 Is a Potent Fungicide That Disturbs Plasma Membrane Integrity in a Sphingolipid Dependent Manner

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Abstract

The peptidomimetic LTX109 (arginine-tertbutyl tryptophan-arginine-phenylethan) was previously shown to have antibacterial properties. Here, we investigated the activity of this novel antimicrobial peptidomimetic on the yeast *Saccharomyces cerevisiae*. We found that LTX109 was an efficient fungicide that killed all viable cells in an exponentially growing population as well as a large proportion of cells in biofilm formed on an abiotic surface. LTX109 had similar killing kinetics to the membrane-permeabilizing fungicide amphotericin B, which led us to investigate the ability of LTX109 to disrupt plasma membrane integrity. *S. cerevisiae* cells exposed to a high concentration of LTX109 showed rapid release of potassium and amino acids, suggesting that LTX109 acted by destabilizing the plasma membrane. This was supported by the finding that cells were permeable to the fluorescent nucleic acid stain SYTOX Green after a few minutes of LTX109 treatment. We screened a haploid *S. cerevisiae* gene deletion library for mutants resistant to LTX109 to uncover potential molecular targets. Eight genes conferred LTX109 resistance when deleted and six were involved in the sphingolipid biosynthetic pathway (*SUR1*, *SUR2, SKN1*, *IPT1*, *FEN1* and *ORM2*). The involvement of all of these genes in the biosynthetic pathway for the fungal-specific lipids mannosylinositol phosphorylceramide (MIPC) and mannosyl di-(inositol phosphoryl) ceramide ($M(IP)_2C$) suggested that these lipids were essential for LTX109 sensitivity. Our observations are consistent with a model in which LTX109 kills *S. cerevisiae* by nonspecific destabilization of the plasma membrane through direct or indirect interaction with the sphingolipids.

Citation: Bojsen R, Torbensen R, Larsen CE, Folkesson A, Regenberg B (2013) The Synthetic Amphipathic Peptidomimetic LTX109 Is a Potent Fungicide That Disturbs Plasma Membrane Integrity in a Sphingolipid Dependent Manner. PLoS ONE 8(7): e69483. doi:10.1371/journal.pone.0069483

Editor: Christopher Beh, Simon Fraser University, Canada

Received March 18, 2013; **Accepted** June 10, 2013; **Published** July 12, 2013

Funding: Funding provided by the Danish Agency for Science Technology and Innovation (FTP 10-084027). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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☯ These authors contributed equally to this work.

Introduction

Infections caused by pathogenic yeast such as *Candida spp*. affect a large number of immunosuppressed patients and are an increasing medical problem [1,2]. Fungal infections are currently treated with one of four major classes of antifungals. Azoles target ergosterol synthesis [3], polyenes bind to ergosterol in the cell membrane and form pores [4,5], echinocandins inhibit cell wall synthesis [6], and 5-fluorocytosine interferes with protein and DNA synthesis [7].

Decreased susceptibility to the most frequently used antifungal, fluconazole, has recently been reported, and the number of nonsusceptible *C. glabrata* isolates from humans is increasing [8,9]. Resistance towards 5-fluorocystosine is also rapidly developing [10]. Polyenes can be toxic [11] and echinocandins have a narrow spectrum of activity [12]. An additional complication in the treatment of nosocomial fungal infections is the frequent formation by fungi of sessile communities called biofilms in association with medical implants [13]. Limited nutrient access leads to slow-growing, antibiotic tolerant cells in biofilms that can serve as a reservoir for infection [14,15]. Most systemic antifungals are fungistatic against yeasts, so they are primarily effective against actively growing cells and have poor activity against cells in biofilms.

The limited number of antifungal classes and drugs with fungicidal properties raises the need for novel drugs with activity against slow-growing and biofilm-forming pathogenic fungi [16,17]. Antimicrobial peptides (AMPs) and modified forms of AMPs offer an attractive alternative to conventional antifungal drugs. AMPs are cationic and amphipathic peptides of 12–50 amino acids that are produced by species in almost every kingdom and phylum of life [18]. The amphipathic structure of AMPs suggests that they might have targets that are different from conventional antifungals [19,20]. The high degradation rate of many natural AMPs can be circumvented by backbone and side chain alterations that create structural analogs that mimics natural peptides [21]. A number of synthesized peptidomimetics have *in vitro* antifungal activity, making these compounds attractive candidates for novel antifungal drugs [22–24].

We tested the antifungal activity of the short, antibacterial peptidomimetic LTX109 (arginine-tertbutyl tryptophan-arginine-phenylethan). LTX109 is based on an Arg-Trp-Arg sequence found in the AMP bovine lactoferricin and was originally developed as an antibacterial [25–27].

We used killing kinetics to describe the antimicrobial effect of LTX109 and investigated its mode of action by measuring transport of H^+, K^+, amino acids and a fluorescent dye across the cell membrane. To uncover potential molecular targets that would explain the fungicidal activity of LTX109, we screened a *Saccharomyces cerevisiae* gene deletion library for mutants resistant to LTX109. Most mutations that led to LTX109 resistance were in genes involved in the synthesis of the sphingolipids mannosylinositol phosphorylceramide, MIPC, and mannosyl di-(inositol phosphoryl) ceramide, $M(IP)_2C$. These results indicate that $M(IP)_2C$ and/or MIPC in the plasma membrane are essential for the action of LTX109.

Materials and Methods

Strains, growth media and antifungal drugs

The S288c *S. cerevisiae* strain M3750 (*MAT***a** *ura3-52*) [28] was used as the reference strain in all experiments unless otherwise indicated, while the barcode-tagged deletion-mutant library was from Johnston and coworkers [29]. Σ1278*b* (10560-2B; *MAT***a** *ura3-52 leu2:hisG his3:hisG*) was used for biofilm susceptibility experiments [30]. Complex YPD medium [31] was used in all experiments except for amino acid release and biofilm where cells were grown in synthetic complete medium [31]. LTX109 (Lytixar; LytixBiopharmaAS, Oslo, Norway) and amphotericin B (Sigma) were dissolved in water and stock solutions were kept at −20°C.

Broth microdilution minimal inhibitory concentrations

Minimal inhibitory concentration (MIC) values were measured under static conditions in polystyrene microtiter plates. Two-fold dilution series of antifungal drug were prepared in fresh YPD medium and distributed to microtiter-plate wells. Overnight cultures of wild type (WT) S288c were diluted and added to antifungal-containing wells to a final concentration of 2×10^5 cells/ml. Growth inhibition was recorded with absorbance at 600 nm after 24 hours at 30°C. The lowest drug concentration resulting in 90% growth inhibition was the MIC. MIC values of LTX109 were determined three times with triplicate measurements, while MIC values of amphotericin B was determined once with triplicate measurements.

Killing kinetics

Overnight cultures of *S. cerevisiae* were diluted in fresh, preheated YPD to 4×10^5 cells/ml and incubated at 30°C with aeration. Exponential growth phase cells were challenged with LTX109 or amphotericin B at concentrations that were five times the MIC. Control samples were treated with water to ensure that cells applied in the time-kill experiment were in exponential growth phase. Samples were taken at the indicated time points, diluted 10-fold, and plated on YPD agar to determine colony forming units (CFUs). The time-kill experiment was conducted in triplicates.

Acidification assay

Glucose-induced acidification was measured as previously described [32] with modifications. Exponentially growing *S. cerevisiae* cells were washed and resuspended in sterile water to a final concentration of 10^8 cells/ml. Cells were subsequently incubated with LTX109 (100 µg/ml) or water (control) for 10 minutes before the assay was initiated by addition of glucose to a final concentration of 2% (w/v). The assay was conducted in triplicate at room temperature with continuous magnetic stirring. The assay was stopped by sampling at indicated time points, followed by immediate centrifugation ($2000\times g$ for two minutes). pH of the resulting supernatants was measured and changes in extracellular H^+ concentration were calculated by applying the obtained values to the equation $pH = -\log [H^+]$.

Potassium release

Exponentially growing *S. cerevisiae* were harvested and resuspended in sterile water as described above. The potassium release assay was initiated by addition of LTX109 to a final concentration of 10 times the MIC. Samples treated with water instead of LTX109 served as control. The assay was stopped by centrifugation of samples ($13{,}000\times g$ for 1 min) at indicated time points. Supernatants were transferred to sterile microtubes for spectrometric analysis. Potassium concentrations were measured with a FLM3 flame photometer (Radiometer). A standard concentration curve was generated from diluted S3336 urine flame standard (Radiometer). For spectrometric analysis, 5 µl of sample was added to 1000 µl of S3336 lithium solution (Radiometer). Experiments were carried out in triplicates at room temperature.

SYTOX Green uptake

SYTOX Green uptake was measured as previously described [33] with modifications. Exponentially growing *S. cerevisiae* cells were centrifuged, washed and suspended in 5 µM SYTOX Green (Life Technologies) to a final concentration of 10^8 cells/ml. LTX109 or water (control) was added to cell suspensions and SYTOX Green uptake was recorded microscopically after 4, 8, 16, 32, 64 and 128 minutes. Fluorescence was recorded with a Nikon Eclipse (Tokyo, Japan) fluorescence microscope equipped with a F36–525 EGFP HC-filter set (AHF Analysentechnik). Experiments were carried out at room temperature. SYTOX green uptake upon LTX109 treatment was observed in three independent experiments.

Amino acid release

Exponentially growing *S. cerevisiae* were harvested and suspended in sterile water or water with 10 times the MIC of LTX109 to a final concentration of 2×10^6 cells/ml. Loss of free amino acids from cells was recorded at room temperature after 16 minutes LTX109 exposure by instant centrifugation and subsequent HPLC of the cell free supernatant. Amino acids were detected and quantified by reverse-phase HPLC using an LKB-Alpha Plus amino-acid analyzer and a mixture of L-α-amino acids, 1 nmol each, as standards. The experiment was repeated three times.

Identification of LTX109-resistant mutants

Haploid knockout mutants of approximately 4000 nonessential genes in the S288c deletion mutant library [29] were pooled. About 10^6 cells from the mutant pool were transferred to YPD agar containing 10 times the MIC of LTX109. After 72 hours at 30°C, 17 colonies were picked from the LTX109 plates. LTX109-resistant clones were identified by PCR amplification and Sanger sequencing of the unique barcode tag of each mutant. PCR templates were DNA from clonal isolates of LTX109-resistant mutants. Primers were 5′-GATGTCCACGAGGTCTCT and 5′-CTGCAGCGAGGAGCCGTAAT. Gene deletions were identified using barcode sequences in the Stanford SGD deletion database.

Spot test

Exponentially growing *S. cerevisiae* cells were diluted to 10^7 cells/ml and 6 µl of a 10-fold dilution series was spotted on YPD agar and YPD agar containing 10 times the LTX109 MIC. Plates were incubated for 24 hours at 30°C and growth results were recorded.

Biofilm susceptibility

S. cerevisiae (Σ1278*b*) cells were grown in Lab-Tek™ Chamber Slide™ System; Permanox® (NUNC, Denmark) [34,35] in 1 ml synthetic complete medium. Cells were initially allowed to form biofilm for 12 hours before LTX109 was added for 5 hour in a concentration of 0 or 70 µg/ml. The biofilm was subsequently stained 15 minutes with Syto 9 (Invitrogen, Irvine, CA) for life cell staining and propidium iodine for dead cell staining before confocal laser scanning microscopy (CLSM). Imaging was carried out using a 63x/0.95NA water immersion lens. CLSM was performed with a Zeiss LSM510 microscope. Staining of biofilm treated with LTX109 was repeated in four independent experiments.

Results

Fungicidal properties of LTX109

We tested the antifungal properties of the peptidomimetic LTX109 on the yeast *S. cerevisiae* using microdilution. LTX109 had antifungal activity against *S. cerevisiae* at 2×10^5 cells/ml with a MIC value of 8 µg/ml compared to 2 µg/ml amphotericin B. Assay to determine the killing kinetics of LTX109 against *S. cerevisiae* revealed rapid and efficient fungicidal properties resulting in a 3-log reduction in viable cells within one hour, while amphotericin B required 90 minutes to achieve a similar fungicidal effect when using drug concentrations in multiples of MIC (Fig. 1). Additionally, LTX109 reduced the yeast population to the detection limit within only 2 hours, an effect that was not achieved by amphotericin B in the first 3 hours of exposure.

Exposure to LTX109 disrupts plasma membrane integrity

The speed with which LTX109 killed *S. cerevisiae* suggested that the compound was acting directly on the plasma membrane. To investigate the effect of LTX109 on plasma membrane integrity, we measured H^+ efflux, ion loss, loss of amino acids and uptake of the fluorophore SYTOX Green across the plasma membrane.

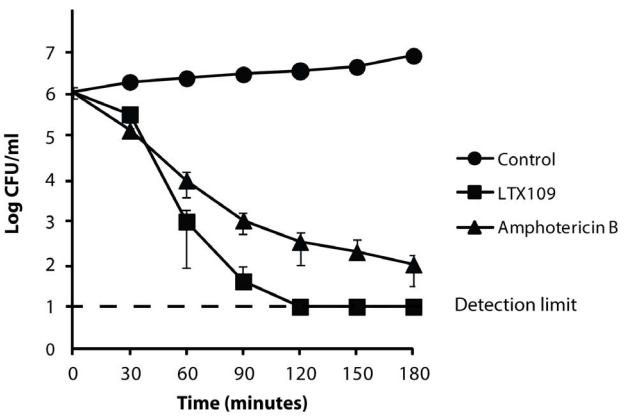


Figure 1. Fungicidal properties of LTX109 and amphotericin B. Time-kill kinetics of exponentially growing yeast cells exposed to water (circles) or five times the MIC of LTX109 (40 µg/ml) (squares) or amphotericin B (10 µg/ml) (triangles). Viability was examined every half hour as CFUs. Each data point is the average of three individual measurements ± standard deviation.
doi:10.1371/journal.pone.0069483.g001

Yeast cells treated with glucose acidify their surroundings primarily by active transport of H^+ by the plasma membrane H^+-ATPase [36]. We found that glucose-induced acidification was completely absent when cells were treated with LTX109 for 10 minutes before glucose addition (Fig. 2A). These results suggested that LTX109 decoupled the plasma membrane potential directly or indirectly by inhibition of e.g. ATP synthesis.

We next tested the loss of potassium from cells treated with LTX109. Potassium release occurred immediately and increased during the first 16 minutes of exposure to LTX109, reaching a steady state that was more than four times higher than the maximum of the untreated control (Fig. 2B). Much of the K^+ that was lost was detected within the first two minutes of challenge with a high LTX109 concentration. These results suggested that LTX109 acted by direct interaction with and disturbance of the plasma membrane rather than through indirect inhibition of metabolism or another intracellular pathway.

To investigate if LTX109 treatment also led to loss of other small molecules, cells were treated with LTX109 for 16 minutes and free amino acids measured in the extract. Yeast cells treated with LTX109 lost substantial amounts of at least 14 different amino acids whereas cells treated with water only leaked aspartate (Fig. 3). The loss corresponds well to the pool of intracellular amino acids found in other experiments [37], suggesting that most if not all free amino acids are depleted from cells treated with LTX109.

We finally investigated if the membrane potential decoupling was a consequence of plasma membrane destabilization by monitoring the uptake of the 600-Dalton nucleic acid stain SYTOX Green. SYTOX is an inorganic compound that fluoresces upon DNA binding and SYTOX Green can only enter a cell and fluoresce if the plasma membrane is compromised [38]. We found that untreated cells were impermeable to SYTOX Green, while cells treated with LTX109 became permeable. The dye was visible in the nucleus of LTX109-treated cells after only eight minutes (Fig. 2C) and fluorescence increased with LTX109 exposure time.

Defects in sphingolipid synthesis lead to LTX109 resistance

To gain further insight into the LTX109 mode of action, we screened a collection of haploid *S. cerevisiae* knockout mutants for LTX109 resistance. We isolated 17 mutants that were resistant to LTX109 at 10 times the MIC on YPD agar. Eight genes conferred LTX109 resistance when deleted (Table 1; Fig. 4A). Six of the identified genes (*SUR1*, *FEN1*, *SUR2*, *IPT1*, *SKN1*, *ORM2*) were involved in the biosynthesis of sphingolipids, which are a major plasma membrane component. Fen1p and Sur2p are involved in synthesis of ceramides, which are precursors for inositol phosphoceramide (IPC), the first complex sphingolipid in the synthesis pathway [39] (Fig 4B). Fen1p elongates long-chain fatty acids that are linked to a sphingoid base to form ceramides [40] and Sur2p hydroxylates dihydrosphingosine (DHS) to form phytosphingosine (PHS) [41], which is the most abundant sphingoid base in yeast ceramides [39]. Sur1p mannosylates IPC to form the intermediate sphingolipid mannose inositol phosphoceramide (MIPC) [42] and Skn1p and Ipt1p have similar functions in the biosynthesis of the terminal sphingolipid mannosyl di-inositol phosphorylceramide ($M(IP)_2C$) [43]. Orm2p is a regulator of the sphingolipid biosynthesis that links the biosynthesis to the regulatory Target Of Rapamycin pathway [44]. Mutants that fail to activate Orm2p have reduced levels of sphingolipids as do *fen1*, *sur1*, *ipt1* and *skn1*

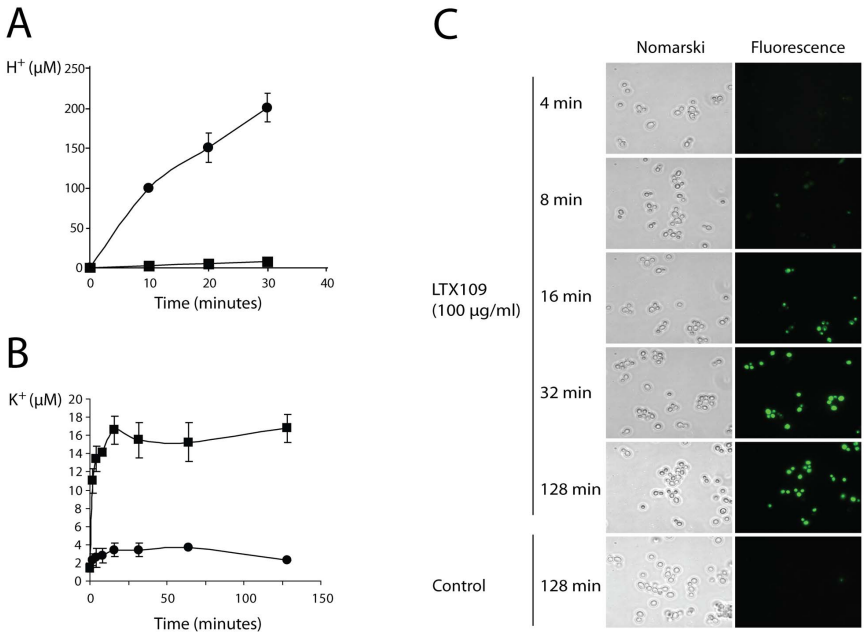


Figure 2. Transport of H^+, K^+ and a fluorescent dye by cells treated with LTX109. (A) Glucose-induced acidification of medium by yeast cells. Exponentially growing *S. cerevisiae* was washed and suspended in sterile water and exposed to 100 μg/ml LTX109 (squares) or water (circles) before glucose addition at time zero. Medium pH was measured and H^+ concentration calculated from pH = −log [H^+]. Each data point is the average of three individual measurements with standard deviations as error bars. (B) Potassium release from yeast cells. Exponentially growing yeast cells were washed, resuspended in water, and challenged with 100 μg/ml LTX109 (squares) or water (circles) at time zero. Potassium release was measured using flame atomic absorption spectrometry in binary increasing intervals. Each data point is the average of three individual measurements ± standard deviation. (C) Nomarski (left) and fluorescent (right) microscopy of SYTOX Green-stained yeast cells. Exponential growing cells were exposed to 100 μg/ml LTX109 and SYTOX Green uptake was monitored. Cells treated with SYTOX Green and 0 μg/ml LTX109 served as control. SYTOX green uptake upon LTX109 treatment was observed in three independent experiments.
doi:10.1371/journal.pone.0069483.g002

mutants [40,43,45,46], suggesting a role of sphinolipids in sensitivity to LTX109.

One LTX109-resistant mutant was affected in the *YSP2* gene, which is involved in apoptosis, and another was affected in *OPI9*.

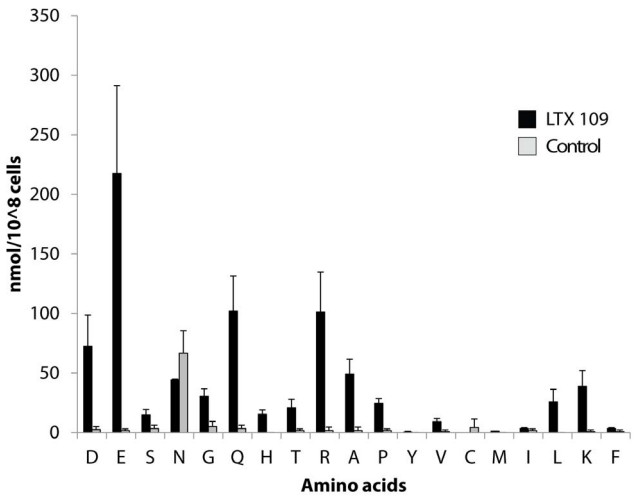


Figure 3. Efflux of amino acids from cells treated with LTX109. Exponentially growing yeast cells were washed, resuspended in water, and challenged with 70 μg/ml LTX109 (black bars) or water (grey bars) for 16 minutes. Amino acids (one letter code) in the extracellular medium were subsequently measured by HPLC. Each data point is the average of three individual measurements ± standard deviation.
doi:10.1371/journal.pone.0069483.g003

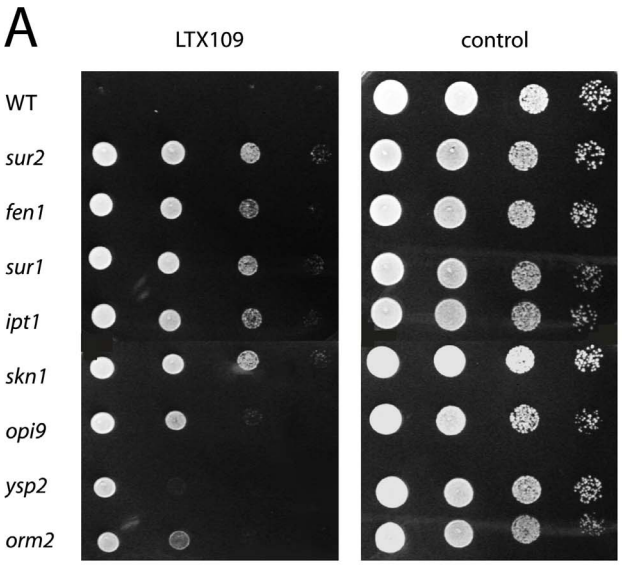
OPI9 has an unknown function but partly overlaps with *VRP1*, which encodes an actin-associated protein with a role in actin filament organization. The *opi9* mutant therefore also has a partial deletion of *VRP1*, so the LTX109-resistance phenotype could be caused by loss of Vrp1p activity. Resistance of each mutant was confirmed by spot-testing diluted yeast suspensions on YPD agar containing LTX109 (Fig. 4A). Five of the mutants affected in sphingolipid biosynthesis showed similar, high resistance towards LTX109 (*sur1*, *fen1*, *sur2*, *ipt1* and *skn1*).

LTX109 efficiently kill S. cerevisiae growing as biofilm

Because S288c is incompetent of biofilm growth [47,48] we used the Σ1278*b* strain background to test the antifungal activity of LTX109 against *S. cerevisiae* biofilm. To visualize the antifungal properties of LTX109, we used CLSM in combination with Syto 9 DNA viability stain and propidium iodide that only penetrates damaged cell membranes. Intermediate (12 h) *S. cerevisiae* biofilm grown in batch culture slides were treated with 10 times MIC LTX109 for 5 hours before LIVE/DEAD staining and CLSM (Fig. 5). The LTX109 treatment killed the majority of the biofilm population as indicated by uptake and staining of dead cells with propidium iodide (Fig. 5), suggesting that LTX109 is also an efficient anti-biofilm agent in addition to its fungicidal activity against planktonic cells in exponential growth phase.

Discussion

This study demonstrated the fungicidal activity of the peptidomimetic LTX109. Antimicrobial peptidomimetics are peptide-like compounds, of which most are bactericides [49–53].



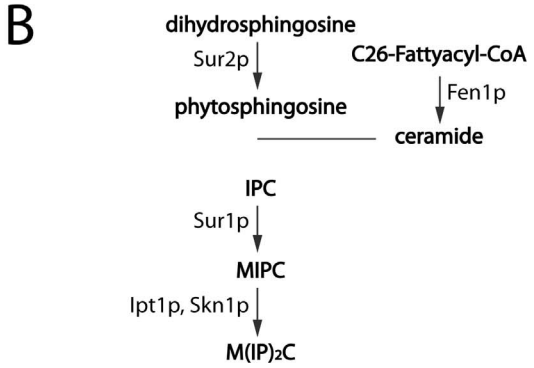


Figure 4. Mutants in sphingolipid biosynthesis are resistant to LTX109. (A) Spot test of wild type (WT) *S. cerevisiae* and eight deletion mutants identified by screening a deletion collection for LTX109 resistance. Exponentially growing yeast was resuspended in YPD to 10^7 cells/ml and serially diluted 1:10. Aliquots (6 µl) were spotted on solid YPD plates containing 70 µg/ml LTX109 (left panel), and without LTX109 (right panel). (B) *S. cerevisiae* sphingolipid biosynthetic pathway.
doi:10.1371/journal.pone.0069483.g004

LTX109 was previously shown to have bactericidal activity [27]. The arginine–tertbutyl tryptophan–arginine sequence of LTX109 makes it amphipathic, with two bulky side groups and two cationic side groups that are proposed to interact with negatively charged bacterial cell membranes [26].

We found similar killing kinetic for LTX109 and the membrane permeabilizing drug amphotericin B, suggesting that the two compounds could have a related mode of action. The rapid uptake of the fluorescent dye SYTOX Green, potassium and amino acid efflux from cells exposed to a high concentration of LTX109 suggest that this drug disturbs the plasma membrane by direct interaction with one or several components in the plasma membrane. Furthermore, inability of LTX109 treated cells to acidify their surrounding media support an effect on the cell membrane.

These results are similar to results with amphotericin B, which also causes yeast cells to inhibit glucose-induced acidification [32] and to release potassium as a consequence of general membrane disorganization [54,55].

The high concentration of drug could have obscured other toxic effects of LTX109 on *S. cerevisiae*, so we cannot exclude that LTX109 has other effects in addition to membrane disruption as previously discussed for peptide drugs [56].

To gain further insight into the mode of function of LTX109, we screened for resistant mutants. Six of eight resistance mutants were affected in sphingolipid biosynthesis, and five of these showed similar, high resistance towards LTX109 (Fig. 4). *fen1*, *sur1*, *ipt1* and *skn1* mutants all have reduced amount of sphingolipids [40,43,45,46] as do mutants that reduce Orm2p activity [44], suggesting an essential role of complex sphingolipids in sensitivity to LTX109. Lack of Sur2p lead to decreased sphinganine hydroxylation, but does not prevent formation of MIPC [57]. Furthermore, the *fen1* mutant produce reduced amount of sphingolipids containing the C26 acyl group [40]. The resistance phenotype of the *sur2* and *fen1* mutants therefore suggested that it is not only the quantity, but also the structural modifications that occur during sphingolipid synthesis that is required for optimal LTX109 activity. The terminal steps of sphingolipid biosynthesis in yeasts are MIPC and $M(IP)_2C$. The fact that these lipids are reduced in the resistant mutants suggests that MIPC and $M(IP)_2C$ are essential for the fungicidal activity of LTX109, either by direct interaction with LTX109 or by interaction with another mem-

Table 1. *S. cerevisiae* genes that confer LTX109 resistance upon deletion.

Deleted gene and function	ORF	Gene product	n
Sphingolipid biosynthesis			
SUR1	YPL057C	Mannosylinositol phosphorylceramide (MIPC) synthase catalytic subunit	2
SUR2	YDR297W	Sphinganine C4-hydroxylase	8
ORM2	YLR350W	Sphingolipid homeostasis. Interacts with serine palmitoyl transferase (SPT)	1
IPT1	YDR072C	Inositolphosphotransferase, involved in synthesis of mannose-(inositol-P)2-ceramide (M(IP)2C)	1
FEN1	YCR034W	Involved in membrane-bound fatty acid elongation up to 24 C (ceramide precursor)	1
SKN1	YGR143W	Involved in the terminal M(IP)C →M(IP)2C process	2
Apoptosis			
YSP2	YDR326C	Mitochondrial protein in programmed cell death.	1
Unknown function			
OPI9	YLR338W	Dubious ORF unlikely to encode a protein. Partly overlaps *VRP1*	1

n, number of mutants identified.
doi:10.1371/journal.pone.0069483.t001

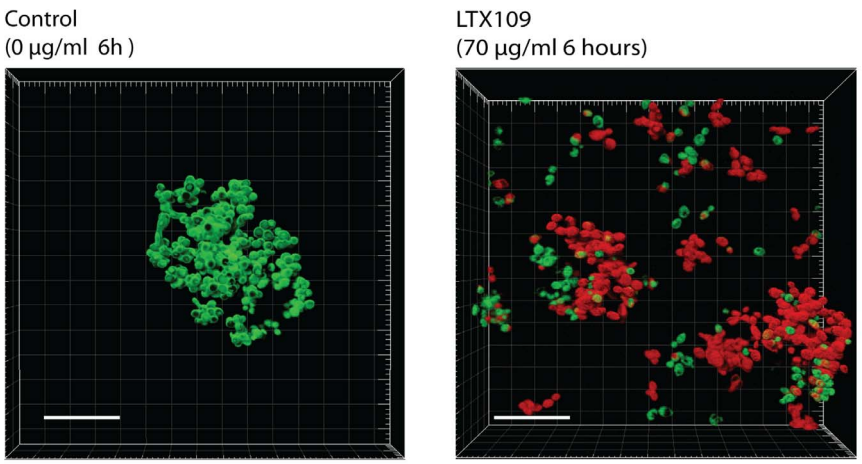


Figure 5. Activity of LTX109 against yeast biofilm. Confocal Laser Scanning Microscopy of *S. cerevisiae* (Σ1278*b*) biofilm. Cells were grown in Lab-Tek™ Chamber Slide™ System; Permanox - (NUNC, Denmark) in 1 ml synthetic complete medium After 12 hours, the cells were exposed to 0 µg/ml LTX109 (control) or 70 µg/ml LTX109 for another 5 hours. The biofilm cells were then stained with Syto9 (green) and propidium iodide (red) LIVE/DEAD stain before confocal laser scanning microscopy. Images are 3D reconstructions of biofilm made from 2 µm thick images in stacks of 20 individual images. CLSM was perform with a Zeiss LSM510 microscope using a 63x/0.95NA a water immersion lens. Life dead staining of biofilm treated with LTX109 was repeated in four independent experiments. White bar is 30 µm.
doi:10.1371/journal.pone.0069483.g005

brane components that is the target for LTX109. It does however seem less likely that a component other than sphingolipids is the target for LTX109 for two reasons, (i) mutants depleted of the target would be expected to appear in the screen for mutants resistant to LTX109. (ii) Alternatively, the target could depend on sphingolipids for optimal activity, be essential for growth and thus not appear in the screen, but then *fen1*, *sur1*, *ipt1*, *skn1* and *orm2* mutants would be expected to have reduced growth rates which they do not (Fig. 4).

Sphingolipids are located primarily in the plasma membrane [58] and are often clustered together with ergosterol in lipid rafts [59]. Sphingolipids are not only a structural component of the cell membrane, but serve vital functions in the heat-shock response, cell cycle arrest, signaling pathways, endocytosis and protein trafficking [60,61]. Fungal sphingolipids are highly similar to each other [62,63], and the biosynthesis of complex fungal sphingolipids is distinctly different from mammals [64]. This makes the fungal sphingolipids attractive antifungal drug targets and several natural compounds with anti-IPC synthase activity have been identified [65–67].

The terminal $M(IP)_2C$ is the major sphingolipid in the fungal plasma membrane [46] and has previously been suggested as a target for the plant defensin *Dahlia merckii* antimicrobial peptide 1 (DmAMP1) [43,68,69]. DmAMP1 is a 50 amino acid amtimicrobial peptide that leads to nonselective passage of potassium, calcium [70] and SYTOX Green [33]. Hence, DmAMP1 and LTX109 could have similar modes of action, although DmAMP1 does not contain the Arg-Trp-Arg sequence that serves as basis for LTX109.

Amphotericin B is currently the last in line treatment option for severe fungal infections [71]. Alternative drug candidates might therefore be developed for treatment in cases where use of amphotericin B becomes limited due to resistance. Biofilm formation on medical devices is a major nosocomial problem and causes multidrug resistance [13]. Only a few of the current systemic antifungals have activity against yeast biofilms [72,73], but often it requires removal of the implant for effective treatment [74]. Peptide antibiotics including LTX109 analogues have been shown to be efficient drugs to kill bacterial biofilm cells [25,50]. This study shows for the first time a peptidomimetic with activity against yeast biofilm. This observation suggests antifungal peptidomimetics with rapid killing kinetics and membrane permeabilizing activities are attractive drugs for yeast biofilm treatment.

In conclusion, we have shown the efficient fungicidal properties of a synthetic peptidomimetic, LTX109, that killed the yeast *S. cerevisiae* with fast killing kinetics and complete eradication of viable cells in exponential growth phase. We found that yeast cells treated with a high concentration of LTX109 became permeable to free amino acids, potassium and SYTOX Green and prevented proton extrusion in response to a pulse of glucose. Fungal susceptibility to LTX109 depended on biosynthesis of sphingolipids. The sphingolipids $M(IP)_2C$ and its precursor MIPC are found in fungal, but not human membranes, making LTX109 and derivatives attractive drug candidates for fungal infection treatment as alternatives to amphotericin B.

Acknowledgments

LTX109 was kindly provided by LytixBiopharma AS, Tromsø, Norway.

Author Contributions

Conceived and designed the experiments: RKB RT BR. Performed the experiments: RKB RT CEL BR. Analyzed the data: RKB RT AF BR. Wrote the paper: RKB RT BR.

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